CYCLIC AMP AND THE

CHLAMYDOMONAS REINHARDTII

CELL DIVISION CYCLE

BY

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STATEMENT

All the research reported in this thesis is original
and my own, except where due acknowledgement is made,
and has not been submitted for any other degree.

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ABSTRACT

Three temperature-sensitive cAMP requiring mutants of *Chlamydomonas reinhardtii* have been isolated. These mutants were obtained from EMS mutagenized cells by selecting for those that were resistant to the phosphodiesterase inhibitor, caffeine, at the permissive temperature of 21°C and were arrested in cell division, but continued to grow, at the non-permissive temperature of 33°C. The isolated mutants were then screened for those which specifically required cAMP for division at 33°C. Processes that required cAMP were identified and studied by addition or denial of cAMP to these mutants at 33°C. The addition of exogenous cAMP or dibutyryl-cAMP (db-cAMP) rescued the cell division cycle (cdc) mutants to division at 33°C, whereas 5'-AMP was not effective.

A new method was developed for measuring nuclear DNA content of these mutants using quantitative fluorescence microscopy of nuclear DNA and was demonstrated to be superior to chemical assays of DNA for analysis of large cells blocked in division. This nuclear DNA fluorescence method indicated that all three mutants were arrested in G1 phase. Arrest in late G1 phase after execution of the commitment to division, which is equivalent to "START" of cell division event in yeast, was indicated by the terminal phenotypes of the arrested cells. Mutant cells were observed using specific antibodies as probes to investigate the cell cytoskeleton. It was found that all three mutants were arrested at the same stage of the cell cycle. The immunofluorescence studies of these mutants using the mitosis-specific monoclonal antibody, MPM-2, which reconizes a family of phosphorylated proteins associated with nuclear envelope in *C. reinhardtii* during metaphase/anaphase, failed to show a positive staining pattern which suggested that the arrest point is prior to metaphase. Observation of cytoskeletal components, β-tubulin, showed that the arrested cells had grown to a large size and become round. Cells had withdrawn their flagella and increased in cortical microtubules. This organization is typical in cells in late G1 phase that have become committed to division. This conclusion is supported by using the mammalian centrosome monoclonal antibody, MPM-13, which detects the basal bodies flagellar root complex in *C. reinhardtii* cell, and antibody against acetylated-tubulin, which is present in axoneme, basal bodies and a subset of cytoplasmic microtubules. These two antibodies labelling showed that the

basal bodies which normally duplicate and migrate to mitotic spindle poles as mitosis progresses, were not yet duplicated. Also the immunofluorescence studies of Ca^{2+}-ATP dependent contractile protein centrin showed that the centrin connector, which normally contracts at preprophase, were still detectable. Late G1 arrest, which was indicated by cytoskeleton configuration, correlated with the phase indicated by nuclear DNA content.

Genetic analysis of these mutants was performed by back crossing them with wild-type to determine the number of mutated genes and to obtain them in the wild-type genetic background. Each mutant contained four mutated genes;

(i) *cdc C* gene; mutation in this gene causes arrest at 33°C under the condition prevailing on the illuminated agar plates, slow growth and smaller colony size, and reduced stationary cell density in liquid medium unless supplemented with cAMP.

(ii) *pot* gene; mutation in this gene enhances the effect of the *cdc C* gene and gives arrest in liquid medium at 33°C. Presence of mutant genes of this type correlated with reduced activity and with *in vitro* thermolability of adenylate cyclase.

(iii) *res* gene; mutation in this gene allows cells that contain the *cdc C* and the *pot* gene to resume cell division in liquid medium at 33°C if supplemented with cAMP. The *res* gene mutation preserves a basal level of adenylate cyclase activity in cells containing the *pot* mutation.

(iv) *caf R* gene; mutation in this gene results in caffeine resistance at 21°C and is not essential for the cdc phenotype.

The presence of *cdc C* and *caf R* genes could be detected directly and linkage between them was measured. Different *cdc C* and *caf R* genes were mutated in the three mutants. Presence of the *pot* and *res* genes was deduced from their phenotypic effects in combination with other genes. The gene combinations that were deduced to be necessary to account for the phenotypes were supported by direct evidence for effect on activity of adenylate cyclase and level of cAMP and by the correlation between observed frequencies of partial phenotypes and their predicted frequencies in tetrad analysis. Full analysis of co-identity of phenotypically equivalent genes was not feasible in the time available.

Defective cAMP-metabolism in these mutants was not caused by phosphodiesterase activity which was normal in all mutants, but could be explained by altered level and thermolability of adenylate cyclase activity. A fall in extractable calcium independent adenylate cyclase activity to zero in mutant cells at 33°C preceeded their division arrest. The rapid *in vitro* thermal inactivation of adenylate cyclase from one of

the mutants indicated that a gene encoding a component of the enzyme has been identified.

To measure cAMP a method for extraction of cAMP was optimized and new procedures for HPLC fractionation were developed. The cellular cAMP content in these mutants correlates with *in vitro* measurements of adenylate cyclase activity. The arrest of cell division in liquid medium correlates with both low adenylate cyclase activity and low endogenous cAMP level, which in arresting cells were less than 5% of wild-type levels. Therefore it is concluded that cAMP is needed for the transition from G1 to S phase in *C. reinhardtii* cell division cycle.

ABBREVIATIONS

5'-AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CaM	calmodulin
cAMP	cyclic adenosine 3',5'-monophosphate
cdc	cell division cycle
cpm	count per minute
DABA.2HCl	diaminobenzoic acid dihydrochloride
DAPI	4',6-diamidino-2-phenylindole
db-cAMP	N^6-2'-O-dibutyladenosine 3',5'-cyclic monophosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	Einstein ($\mu Em^{-2}s^{-1}$; light intensity)
EDTA	ethylene-diamine-tetra-acetic acid
EGTA	ethylene glycol bis(β-aminoethylether) N,N,N',N'-tetraacetic acid
EMS	ethyl-methane-sulphonate
FITC	fluorescein isothiocyanate
fl	femtolitre
g	gram(s)
GTP	guanosine triphosphate
h	hour
l	litre
μ	micro
m	milli
M	molarity
MCV	mean cell volume
min	minute
ml	millilitre
mt	mating type

MTSB	microtubule stabilising buffer
OD	optical density
p	pico
PAR	photosynthetically-active-radiation, i.e., light: 400-700 nm
PBS	phosphate buffered saline
PCA	perchloric acid
PFA	paraformaldehyde
PI	propidium iodide
PIPES	piperazine-N,N'-bis(2-ethane-sulphonic acid)
PMSF	phenylmethylsulphonyl fluoride
RBC	red blood cell
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
UV	ultra violet
wt	wild-type

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INTRODUCTION

SECTION 1. INTRODUCTION

The cell division cycle

The cell cycle is a process that is responsible for the duplication of a cell. Each new cell receives an exact replica of the hereditary information of the mother cell. The modern concept of the cell cycle can be considered to have begun when Howard and Pelc (1953) discovered that DNA replication occurred in a discrete period during interphase so leading to the definition of four phases of the cell cycle: Gap 1, S (synthesis of DNA), Gap 2 and Mitosis. Most cells first grow and then, if conditions are favourable and a necessary minimum size has been attained, become committed to division. However if conditions are unfavourable they enter a specialized resting state that is often called G0.

Once committed to division in late G1 phase, the cell passes through a sequence of four major phases; S, G2, mitosis and cytokinesis. The first three cell cycle phases, G1, S and G2 are known as interphase. G1 phase was originally defined as a gap interval between mitosis and DNA synthesis. It is now recognized as a period of biochemical activity resulting in growth. S phase is a period of DNA synthesis during which the chromosomal material is replicated precisely in a period of a few hours. G2 phase is a period during which there may be assembly of special structures required for mitosis and is very brief in *Chlamydomonas*. Mitosis is a period during which the replicated chromosomes are apportioned between two daughters nuclei. Cytokinesis usually follows immediately after mitosis and results in division of the cytoplasm to form two separate and complete new cells.

Cell cycle control

Much current work is now focusing on the molecular mechanisms that regulate progress through the cell cycle. It has been established in yeasts that cell division is coordinated with growth by requirement of minimum cell size for progress through key rate limiting control points in the cell cycle. These two processes must be regulated in order to produce cells of a consistent size and an organism with a defined form and coherent structure. During the last few years, great progress has been made in attempting to explain the sensor mechanisms which lead a cell to multiply instead of being quiescent and also the mechanisms by which cell cycle processes, especially nuclear division, are initiated.

Recent advances in understanding of the cell cycle have been promoted by genetic analysis of yeasts and biochemical analysis of oocytes. The genetic work was initiated in budding yeast by Hartwell (1978) and later in the fission yeast by Nurse and his colleagues (1980). The biochemical approach was first applied to mollusc and amphibian oocytes. The early genetic evidence indicated that the initiation of each cell cycle event is dependent on the completion of the preceding steps (Hartwell, 1978). Very recently the evidence from both yeasts and oocytes has converged to suggest that the control of mitosis in all major eukaryotes involves a group of highly conserved control proteins (reviewed by Nurse, 1990).

During the last few years, great progress has been made in identifying the proteins that regulate the initiation of mitosis following the initial detection of their phenotypic effect in the budding yeasts, *Saccharomyces cerevisiae* (Hartwell, 1978; Pringle and Hartwell, 1981) and the fission yeast, *Schizosaccharomyces pombe* (Nurse and Fantes, 1981; Lee and Nurse, 1988). A large collection of cell division cycle (CDC) mutants that arrested at a specific point in the cell cycle were isolated initially by Hartwell's group working with budding yeasts (Hartwell et al., 1973). Analysis of these mutants and those in other fungi by cloning and sequencing is increasingly providing information concerning the genes and gene products whose activities are necessary for the crucial cell cycle transitions.

Diverse cells such as those of mammals and yeasts appear to have a common key event that terminates G1 phase. Genetic studies in the budding yeast, *S. cerevisiae*, have identified this primary regulation step as "START" (Reid and Hartwell, 1977; Pringle and Hartwell, 1981). In the budding yeasts, as well as fission yeasts, cells have to reach a minimal cell size before passage through START can occur (Hartwell and Unger, 1977). This regulation point represents commitment to enter the proliferative cell cycle, i.e., to execute the G1/S phase transition and progress with little or no further requirement for growth through G2 and M. In addition, START is also the point where cells respond to external signals. Cells are unable to pass START if they are growing very slowly or in the presence of mating pheromone (Pringle and Hartwell, 1981), they thus abstain from division and are available for mating.

In the fission yeast, *S. pombe*, the second regulatory point between G2 and mitosis has a major role in linking cell division to the attainment of adequate cell size. In medium allowing rapid growth, the cell size required for initiation of mitosis is high and the

resulting daughters are large enough in their next cell cycle to execute START after a minimum time in G1. Nurse and his colleagues identified one gene, referred to as *cdc2*$^+$, that was required for both START and the G2/M transition (Hayles and Nurse, 1986). The *cdc2*$^+$ and *CDC28* genes are functionally interchangable. *CDC28* can rescue the *S. pombe* *cdc2* mutation (Beach et al ., 1982) and a *cdc2*$^+$ intronless gene can rescue *CDC28* mutations of *S. cerevisiae* (Booher and Beach, 1986). Both *CDC28* and *cdc2*$^+$ genes were found to share considerable sequence homology of the predicted polypeptide which resembled known protein kinases and the *cdc2*$^+$ *and CDC28* products were confirmed to have this activity by *in vitro* assay (Reed et al., 1985; Simanis and Nurse, 1986; Brizuela et al., 1987). These studies were extended to human cells by Lee and Nurse (1987), who were able to isolate a human homologue of the *cdc2*$^+$ gene by using a human cDNA library to recover a single gene that could complement mutation of the *cdc2*$^+$ gene in *S. pombe*. They found that the product of this gene was highly homologous to that of *cdc2*$^+$ and *CDC28* genes. This evidence suggested that some of the key proteins and therefore perhaps some of the regulatory mechanism of cell division in eukaryotes are highly conserved.

cdc2$^+$ and _CDC28_ gene products

The products of the *cdc2*$^+$ and *CDC28* genes are commonly referred to as p34^{cdc2} in recognition of their 34 kD molecular weight and coding by the *cdc2*$^+$ gene of *S. pombe* (Simanis and Nurse, 1986; Lee and Nurse, 1987). A functional similarity of *cdc2*$^+$ and CDC28 was indicated by evidence that both are needed for the START (Piggot and Carter, 1982; Nurse, 1985). However Reed et al. have maintained for nearly a decade that the CDC28 of budding yeast acts only at START but not at M, while the onset of mitosis may be carried out by some unidentified homologue of CDC28 . Interestingly, in *S. pombe* the *CDC28* gene can carry out all the functions of *cdc2*$^+$ therefore directly indicating a functional equivalence (Beach et al., 1982) and very recently Reed's group have come to accept a mitotic function for p34^{CDC28} (Reed and Wittenberg, 1987).

In proliferating cells of *S. pombe* (Simanis and Nurse, 1986) and Hela (Draetta and Beach, 1987), the level of p34^{cdc2} was found to be relatively constant throughout the cell cycle. It is possible that p34^{cdc2} regulates START by changes in its activity rather than its level (Simanis and Nurse, 1986; Mendenhall et al, 1987). Several genes that regulate p34^{cdc2} function have been identified. The *cdc25* gene although not a phosphatase is

required for removal of phosphate from p34^{cdc2}, which is an essential step in kinase activation (Russell and Nurse, 1986; Dunphy and Newport, 1989; Gautier et al., 1989). It is likely that the activity of *cdc25* is rate-limiting for entry into mitosis (Russell and Nurse, 1986; Moreno, Russell and Nurse, 1990).

The action of *cdc25*$^{+}$ is inhibited by the product from the gene identified as *wee1* which has the opposing effect of delaying entry into mitosis. The activity of *cdc25* promotes mitosis whereas that of *wee1* retards it. Overexpression of *cdc25*$^{+}$ in a strain *wee1*$^{-}$ mutant causes premature, lethal, entry into mitosis. Overexpression of yeast *wee1*$^{+}$ in a fission yeast *cdc25*$^{-}$ mutant strain produced a mitotic block and G2 arrest (Russell and Nurse, 1987a). These results indicate that two separable sets of regulatory molecules operate at the two main cell cycle control points at START and the initiation of mitosis. Even though it has been suggested that the interaction between *wee1* and *cdc25*$^{+}$ gene products control entry into mitosis, it is not yet clear what causes the changes in ratio of these activities that triggers the mitotic events. There is biochemical evidence that mitotically active p34^{cdc2} is dephosphorylated at tyrosine 15 and since the inhibitory *wee1* encodes a protein kinase it could act by phosphorylating p34^{cdc2} (Featherstone and Russell, 1991). However, the activator *cdc25* has no homology with any known phosphatase although its activity *in vivo* is necessary for the dephosphorylation and activation of p34^{cdc2} (Gould and Nurse, 1989). The sequence of *wee1* indicates similarity with serine/threonine kinase and therefore could be considered unlikely to phophorylate tyrosine but very recently Featherstone and Russell (1991) have attributed tyrosine kinase activity to the *wee1* protein. However the relationship of *wee1* protein to p34^{cdc2} is not fully resolved because *in vitro* p34^{cdc2} does not act as substrate for *wee1*. This may indicate that special conditions occur *in vivo* but have not been duplicated *in vitro*. It seems more likely that *wee1*does not act directly on p34^{cdc2} but instead may activate another protein that exerts on inhibitory effect on p34^{cdc2}.

There is genetic evidence that the activity of the *wee1*$^{+}$ gene product is inhibited by the *nim1*$^{+}$ gene product (Russell and Nurse, 1987b). The products of both genes show homology to known protein kinases. These observations suggest that the regulation of mitosis involves changes in the pattern of protein phosphorylation.

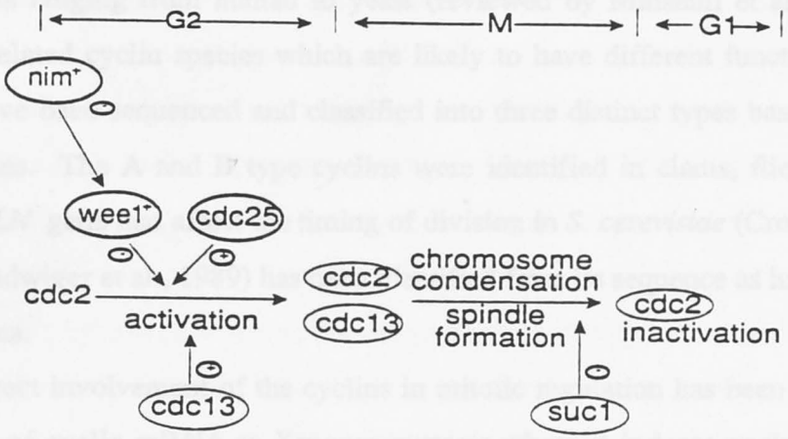
In fission yeast, two other proteins that associate with p34^{cdc2} have been identified as involved in p34^{cdc2} function in initiation of mitosis. One of them is the *cdc13*$^{+}$ gene which encodes a protein with sequence similarity to cyclins (Booher and Beach, 1988;

Hagan et al., 1988; Solomon et al., 1988). Analysis of *cdc13* mutant suggests that $cdc13^+$ is required at two stages during mitosis (Hagan et al.,1988; reviewed by Moreno, Hayles and Nurse, 1989). It is firstly required for $p34^{cdc2}$ protein kinase activation at the initiation of mitosis and must also be present in association with $p34^{cdc2}$ during formation of the mitotic spindle. Cells with temperature-sensitive *cdc13* mutations arrest with condensed chromosomes as in metaphase, but possess the interphase cytoplasmic array of microtubules typical of G2. Mitotic spindles cannot be generated in these cells (Hagan et al., 1988). Overexpression of $p34^{cdc2}$ allows a *cdc13* ts-mutant to grow at its restrictive temperature (Booher and Beach, 1987) and the deletion of this gene results in arrest at late G2 with low $p34^{cdc2}$ protein kinase activity (Booher and Beach, 1987; Hagan et al., 1988).

The other protein that is physically associated with $p34^{cdc2}$ is $p13^{suc1}$, which is encoded by the $suc1^+$ gene (Brizuela et al., 1987). Cells with the $suc1^+$ gene deleted are arrested at the anaphase-telophase transition and retain high levels of $p34^{cdc2}$ kinase activity typical of metaphase (Moreno et al., 1989). This result indicates that $p13^{suc1}$ function is required at a late stage of mitosis.

Study in fission yeast suggests that it is likely that the timing of mitosis is regulated by a network of genes including of $cdc25^+$, $wee1^+$, $nim1^+$ that act upon the $cdc2^+$ gene product. Operation of this network regulates the timimg of activation of the $cdc2^+$ gene function which leads to mitotic initiation. The $cdc13^+$ gene product is also required in this activation but does not influence its timimg (Moreno, Hayles and Nurse, 1989). A number of substrates are phosphorylated by this kinase *in vitro* but there is still uncertainty about which *in vivo* substrates lead to chromosome condensation and mitotic spindle formation. Exit from mitosis and re-entry into interphase requires $p34^{cdc2}$ kinase inactivation brought about by $p13^{suc1}$ activity. The proposed model for cell cycle regulation with emphasis on mitosis is shown in Fig. 1.1.

Fig. 1.1 A model for the induction of mitosis in the fission yeast. Genes products are shown as stimulating (+) or inhibiting (-) the activity of the other gene products (adapted from Murray and Kirscher, 1989; Mereno, Hayles and Nurse, 1989).



The conserved cell cycle

In the last few years the study of eukaryotic cell cycle control has revealed a universality of some regulatory components. Much biochemical evidence from amphibian oocyte division has implicated cyclins as major regulators of nuclear division in egg cells (Minshull et al., 1989). Cyclins were first discovered in fertilized sea urchin eggs and clam oocytes (Evans et al., 1983; Swenson et al., 1989). Later they were identified in diverse organisms ranging from human to yeast (reviewed by Minshull et al., 1989). There are several related cyclin species which are likely to have different functions. Several cyclin genes have been sequenced and classified into three distinct types based on their sequence similarities. The A and B type cyclins were identified in clams, flies and frogs, and the *WHI1/CLN* gene that affect the timing of division in *S. cerevisiae* (Cross, 1988; Nash et al., 1988; Hadwiger et al., 1989) has been identified from its sequence as having homology with the cyclins.

Direct involvement of the cyclins in mitotic regulation has been demonstrated by the injection of cyclin mRNA to *Xenopus* extracts where it induces nuclear division while its removal by proteolysis causes division arrest (Minshull et al., 1989; Murray and Kirschner, 1989). Both cyclins A and B associate with the $cdc2^{+}$ gene product. The study in clams has shown that cyclins A and B form complexes with $p34^{cdc2}$ that exhibit kinase activity (Draetta et al., 1989). The association at mitosis between cyclin B and $p34^{cdc2}$ has been demonstrated in human cells, fission yeast, clam, starfish, sea urchin and *Xenopus* eggs (reviewed by Minshull et al., 1989).

In budding yeast, the *CLN* gene group (*CLN1,2,3*) was identified as involved in progress from G1 to S phase and proved to encode proteins with some homology to the cyclin B class that act at mitosis. Disruption of all three *CLN* genes together results in G1 arresting while disruption of each *CLN* gene alone has little effect on growth. The *CLN1* and *CLN2* genes were identified as suppressors of the *CDC28* gene (Hadwiger et al., 1989) and the *CLN3* (similar to *DAF1* and *WHI1*) was identified as the gene that causes small cell size due to shortened G1 phase (Sudbury et al., 1980; Cross, 1988; Nash et al., 1988). The *CLN2* gene product accumulates during G1 and binds to $p34^{CDC28}$ forming an active histone H1 kinase complex (Wittenberg et al., 1990). Among these genes, the *CLN3* gene shares some sequence homology with the mitotic cyclin (Nash et al., 1988). Thus the cyclin-like proteins that act at G1 in budding yeast are related to others that have

been identified by their involvement in mitosis. The yeast CLN proteins seems to be required in late G1 at START and Reed's group have chosen to classify all the cyclins that act in G1 phase as class A cyclins. In fission yeast, *cdc13*$^{+}$ gene encodes a protein with sequence similarity to cyclins B proteins (Booher and Beach, 1988; Hagan et al., 1988 and Solomon et al., 1988). Certainly the *cdc13*$^{+}$ gene is necessary for mitosis and is required for activation of the p34^{cdc2} protein kinase. The induction of M phase in oocytes also coincides with protein kinase activation.

Another gene implicated in M phase control in multicellular eukaryotes is the Drosophila gene *string*, which has extensive similarity in sequence with *cdc25*$^{+}$ and can complement a *cdc25* inactivation in *S. pombe* (Edgar and O'Farrell, 1989). Deletion of this gene leads to arrest of cell division with a phenotype similar to that observed in *cdc25* mutants of fission yeast. Significantly the level of *cdc25* expression seems to influence the timing of mitosis in a way that suggests its level may be a regulator of mitosis.

The second form of evidence relating to the nature of mitotic control systems derives from biochemical characterization of the maturating promotion factor (MPF) which was identified by studies of oocytes from *Xenopus* and starfish (Masui and Markert, 1971; Gerhart et al., 1984). MPF was described a decade ago as an activity present in metaphase cytoplasm of maturing oocytes and unfertilized eggs that could cause immature oocytes arrested in G2 to undergo maturation via meiotic nuclear division to form haploid eggs (Masui and Markert, 1971; Smith and Ecker, 1971). Microinjection of active MPF into *Xenopus* oocytes arrested just before meiosis, leads to the induction of nuclear division (Wu and Gerhart, 1980; Lohka and Maller, 1985).

Biochemical evidence suggested that MPF was a protein kinase or an activator of protein kinase (Maller and Kreb, 1980). Studies of amphibian and starfish oocytes showed that the appearance of active MPF correlated with the increase in cAMP-dependent protein kinase during the hormone-induced maturation and also the injection of MPF into recipient oocytes resulted in the increase in protein phosphorylation (Maller et al., 1977; Doree et al., 1983). Extensive purification has now revealed that MPF contains a cyclin and a homologue of p34^{cdc2} which is responsible for the protein kinase activity (Labbe et al., 1989).

An important question that was asked during the early characterisation of MPF was whether MPF might be related to any other known cell cycle control elements. This is of particular interest because genetic work in the fission yeast had established that the

product of the *cdc2*$^{+}$ gene was a central regulator of the G2/M transition. All known homologues of p34^{cdc2} share an identical 16 amino acid region with the sequence EGVPSTAIREISLLKE (PSTAIR sequence) that is perfectly conserved from yeast to human but which is unrelated to any general consensus sequence for protein kinase (Lee and Nurse, 1988). It is relevant to the present investigation of a plant cell that a homologue of p34^{cdc2} has also been found in plants and is ubiquitously detected in all of the major plant taxonomic groups including green, brown and red algae (John et al., 1989).

In the highly purified MPF, activity was found to be associated with two proteins; the 34 kD and 47 kD proteins (Lohka et al., 1988). This purified preparation contains a protein equivalent to p34^{cdc2} that is recognized by the anti PSTAIR antibody and co-migrates with authentic human p34^{cdc2} (Gautier et al., 1988). This result indicated that p34^{cdc2} was a component of MPF and therefore that the factor is itself a protein kinase as well as potentially capable of activating other enzymes by phosphorylation.

The p34^{cdc2} component of MPF is at a relatively constant level but its activity oscillates during the oocyte division cell cycle. It appears to be activated just prior to mitosis and inactivated before the metaphase-anaphase transition (Gerhart, 1984). The mechanisms that result in this cycle of activation and inactivation are being investigated and changes in phosphorylation and dephosphorylation of the protein are being implicated. There is also evidence that cyclin is necessary for the appearance of MPF activity and the disappearance of MPF activity is due to destruction of cyclin (Minshull 1989; Murray and Kirshner, 1989). Cyclin was found to be associated with p34^{cdc2} in the high molecular weight complex of histone H1 kinase and in MPF (Draetta et al., 1989; Labbe, 1989b). The activity of the p34^{cdc2} kinase requires cyclin and there is direct evidence that inactivation of *cdc13* in fission yeast prevents the activation of p34^{cdc2}. According to this view, destruction of cyclin would directly inactivate mitotic protein kinase and there is indeed a destruction of cyclin in late mitosis. The ubiquitin system has been identified as involved in cyclin breakdown (Glotzer, Murray and Kirschner, 1991) however the system that triggers this pathway is not clear. Several other proteins are known to be degraded at the end of mitosis for instance topoisomerase II (Earnshaw and Heck, 1989) and thymidine kinase (Sherley and Kelly, in prep., reviewed by Earnshaw and Heck, 1989). The identification of mutants in budding yeast that block sister chromatid separation at the metaphase-anaphase transition provides more information that

may help in understanding this transition. These mutants are highly sensitive to caffeine, which is a known phosphodiesterase inhibitor and this may indicate over production of cAMP in the mutants and by implication a requirement for low levels of this nucleotide in mitosis. The level of cAMP is indeed reported to decrease transiently at the metaphase-anaphase boundary in fission yeast (Hirano et al., 1988). These results suggest that cAMP levels may be an important controlling element in terminating mitosis.

Biological functions of cAMP

A major objective of the research described in this thesis was to investigate whether cAMP had any direct role in plant cell division. This possibility was considered sufficiently plausible to warrant investigation because of evidence in budding yeast that mutations that affect the activation of a cAMP-dependent protein kinase resulted in cell cycle arrest (Matsumoto et al., 1982). This evidence will be discussed in more detail later but it may be appropriate now to very briefly indicate the range and importance of functions that have been attributed to cAMP in eukaryotes.

One of the longest-known functions of cAMP is the control of carbohydrate metabolism in mammals. The cyclic nucleotide here acts as an activator of the protein kinase that activates the phosphorylase kinase which activates glycogen phosphorylase. It is intriguing that there is an echo here of the role of cAMP in carbon metabolism in bacteria where again cAMP is necessary for the synthesis of many induced enzymes essential for the fermentation of sugars (Pastan and Perlman, 1972). It is not clear whether there is any parallel in plant carbon metabolism. An essential function of cAMP in control of Cl ion transport has been indicated recently by the finding that failure of this cAMP control is a causal agent in cystic fibrosis (Drumm et al., 1990).

An even more important function of cAMP in animals is to act as a "secondary messenger" which mediates the action of rapidly acting hormones such as secretin, oxytocin, adrenalin, nonadrenalin, vasopressin, ACTH, TSH and glucagon (Jost et al., 1971). The hormone binds to a specific receptor protein in the cell membrane and this activates adenylate cyclase enzyme that is physically associated with the receptor. The resulting cAMP which is released inside the cell acts as a secondary messenger by binding to the specific site on the regulatory subunits of a cAMP-dependent protein kinase. This protein kinase then becomes activated and capable of phosphorylating other key enzymes such as phosphorylase and lipase which result in modifying the pattern of carbohydrate or lipid metabolism (Nimmo and Cohen, 1977).

In the slime mould, *Dictyostelium discoideum*, cAMP has been shown to function as an aggregation factor, or pheromone, during the amoeboid stage of development (Gerisch and Malchow, 1976).

cAMP has been suggested to be involved in morphogenic development in a variety of fungi. In the dimorphic fungus, *Candida albicans*, there is a six fold decrease in the

intracellular level of cAMP during the transition from yeast-like form to filamentous hyphal form, and treatments that prevent this decline in cAMP block hyphal formation (Egidy et al., 1989). In a colonial fungus, *Neurospora*, the *crisp-1* mutants have been reported to have reduced adenylate cyclase activity and low endogenous levels of cAMP. Exogenous cAMP or dibutyryl cAMP partially correct the aberrant morphology of this mutant (Torenzi et al., 1974, 1976).

A possible role of cyclic nucleotides in the control of cell division has been suggested by a number of studies with synchronously growing mammalian cells. Studies in 3T3 swiss mouse fibroblast, which were synchronized by addition of serum or by trypsinization, demonstrated that the mitotic index peak coincided with a decreased in cAMP level (Burger et al., 1972). In addition, the indication of decreased cAMP levels during mitosis and relatively higher levels in G1 were presented in the studies of Chinese hamster ovary and Chinese hamster V_{79} cells (Sheppard et al., 1972; Russell et al., 1975). Although cell types and synchronization procedures were often different, elevated levels of cAMP during G1 and decreased levels of cAMP during mitosis are most commonly reported. However there are two difficulties with interpreting data from synchronized mammalian cells. One is that the treatment for synchronization will have interrupted growth and it is possible that the changes in cAMP levels are in response to this perturbation and are not a normal part of the cell cycle. The other difficulty is in assessing how essential for division are any changes in cAMP that may occur in particular phases. Such changes may be consequences of cell cycle progress rather than determining controls and they may play no essential part. These objections do not arise if the effects of mutations that specifically affect cAMP can be studied. This approach has been adopted in yeast and was the approach used in the research described in this thesis.

In budding yeast, it has been postulated that there are two classes of genes involved in the control of START which control entry into the two alternative developmental programmes that can occur in haploid cells at the time of START (Reed, 1980). One possible programme is a response to the presence of mating pheromone, which prevents start of division and leads to conjugation with a cell of opposite mating type. Alternatively, commitment to division can occur by completion of START, then the cell can not be arrested by pheromone and will produce daughter cells. START genes of class I are responsible for commitment to the division sequence of events. When mutated they can

result in failure to complete START and therefore the accumulation of an unbudded population in which conjugation can occur and growth can continue. Genes of class I include *CDC28*, *CDC36*, *CDC37* and *CDC39*. START genes of class II may be only indirectly related to cell division and are responsible for maintenance of an adequate growth rate that allows cells to approach and pass through START. Mutation in these genes results in phenotypes similar to the condition of cells starved for some nutrients. Mutation of class II genes causes failure to retain the ability to conjugate because of restricted growth and these mutant cells can neither conjugate nor divide. Genes that cause class II arrest include *CDC19*, *CDC33*, and genes *CDC25* and *CDC35* that are implicated in adenylate cyclase control.

cAMP has been shown to be essential for cell proliferation in budding yeast. Temperature sensitive mutations in genes affecting the cAMP pathway have been classified into three groups by complementation tests; two groups of recessive mutants, *cyr1* and *cyr2*, and a group of dominant mutants, *CYR3*. All these mutants were slow growing and arrested in G1 phase as unbudded, uninucleate cells when shifted to restrictive temperature in the absence of external cAMP (Matsumoto et al., 1982, 1983, 1984; reviewed by Matsumoto, 1986). The *cyr1* mutants are believed to be altered in the structural gene for adenylate cyclase because of the thermolability of adenylate cyclase in a temperature sensitive *cyr1* mutant and the proportional changes of adenylate cyclase activity in tetraploid strains carrying different dosages of *cyr1* gene (Matsumoto et al., 1984, 1985). The *CYR1* gene is considered to be located in the gene previously known as *CDC35*. They map to the same locus and share the same properties of low intracellular cAMP levels at restrictive temperature, low adenylate cyclase activities and they possess thermolability of adenylate cyclase activity (Boutelet et al., 1985; Casperson et al., 1985). The yeast *tsm0185* gene has also shown common identity with *CYR1* gene by mapping to the same locus, causing deprivation of adenylate cyclase activity in mutant strains and the ability of the cloned wild type *tsm0185* gene to complement the *cyr1* mutation (Masson et al., 1984).

Evidence that a major mode of action of cAMP is by activation of protein kinases, comes from the detection of *cyr2* and *CYR3* mutants. These mutants have normal adenylate cylase and phosphodiesterase activities but show abnormalities in cAMP-dependent protein kinases (Matsumoto et al., 1982 and 1983; Uno et al., 1984). The *cyr2* mutants were found to be altered in the catalytic subunit of protein kinases but later it was found that structural

genes for the catalytic subunits of the cAMP-dependent protein kinase in yeast consist of three members; *TPK1*, *TPK2* and *TPK3* (alleic to *SRA3*). Neither single nor double disruption of *TPK* genes have growth defective phenotypes therefore the *CYR2* is probably not one of the *TPK* genes but possibly encodes a gene product that modifies all three catalytic subunits (Toda et al., 1987). The *CYR3* mutant was found to be altered in the regulatory subunit of the same enzyme (reviewed by Gibbs and Marshall, 1989). Cells containing *cyr2* and *CYR3* mutations may produce a normal level of cAMP but growth of the mutant cells will be limited without supplementing cAMP, because a larger concentration of cAMP is required for the activation of the mutant protein kinases enzyme, judging from the increase in activation constant of the mutant enzymes measured *in vitro* (Uno et al., 1983 and 1984).

A number of suppressor mutants were isolated as revertants from the cAMP-requiring mutants. Suppressors are secondary mutations located in different loci that involve some common steps or compensatory pathways and result in a total or partial restoration of the function lost due to a primary mutation. One such suppressor was the *bcy1* mutation (allelic to *SRA1*) which was isolated as a revertant of *cyr1* (adenylate cyclase) mutants (Matsumoto et al., 1982). The *bcy1* mutants had normal levels of adenylate cyclase and phosphodiesterase activities but the regulatory subunit had extremely reduced cAMP binding activity and gave activation of protein kinase in the absence of cAMP. Biochemical evidence showed that the mutant had a high level of cAMP-independent protein kinase, thereby partially compensating for the lack of the cAMP dependent activity. Double mutants of *cyr1*, *cyr2* or *CYR3* (adenylate cyclase or kinase component) with *bcy1* are able to grow in the absence of cAMP because cells have some active cAMP-independent protein kinase (Matsumoto et al., 1982).

The *ppd1* mutant was isolated as a suppressor of the *cyr2* (kinase catalytic subunit) mutation (Matsumoto et al., 1985). The *ppd1* mutant is deficient in one of the three phosphoprotein phosphatases, which are found in yeast wild-type cells (Wingender-Drissen et al., 1983) and the *ppd1* mutation results in decreased dephosphorylation of cellular proteins which have been phosphorylated by cAMP-dependent protein kinase. Therefore suppressor activity of the *ppd1* mutation may be exerted by the retention of critical levels of phosphorylated proteins when rates of phosphorylation have been reduced by the *cyr2*

mutation that is necessary for activation of the catalytic subunit of the cAMP-dependent protein kinase (Toda et al., 1987).

In budding yeast, cAMP is degraded by two phosphodiesterase enzymes encoded by the genes *PDE1* and *PDE2* (allelic to *SRA5*) for low and high-affinity cAMP phosphodiesterases, respectively (Londesborough and Suoranta, 1983; Sass et al., 1986; Nikawa et al., 1987). The *pde1* mutant was isolated as a suppressor of the *CYR3* mutation, which causes low affinity for cAMP in the regulatory subunit of cAMP-dependent protein kinase. The *pde1* mutation partially inactivates the low-affinity (high Km) cAMP phosphodiesterase and confers high intracellular cAMP levels (Uno et al., 1983). Since the *CYR3* mutants require a high level of cAMP for activation of cAMP-dependent protein kinase, a mutation such as *pde1* that results in the over production of cAMP can suppress the *CYR3* phenotype. The other type of *CYR3* suppressor, the *IAC* mutant was isolated with the characteristics of very high adenylate cyclase activity and accumulation of high intracellular cAMP levels in spite of normal levels of phosphodiesterase activity. This mutant therefore accumulates high intracellular cAMP levels. The *IAC.pde1* double mutants accumulate an extraordinarily large amount of cAMP (Matsumoto et al., 1985). The cAMP levels accumulated in *pde1.CYR3* and *IAC.CYR3* double mutant cells may be enough to activate altered cAMP- dependent protein kinase of the *CYR3* strain (Matsumoto et al., 1986) and restore cell growth and division.

In mammalian cells some hormones exert their actions by stimulating a receptor to activate the G-protein (*ras* protooncogene product) which then binds GTP and in that form can activate adenylate cyclase activity (Gilman, 1987; Barbacid, 1987; Bishop, 1987; Hanks et al., 1988). The adenylate cyclase in budding yeast is also localized at the plasma membrane and is composed of at least two protein components; one mediates catalytic activity and the other is responsible for its regulation by guanine nucleotides in the manner similar to the mammalian enzyme (Casperson et al., 1983). Genes homologous to mammalian *ras* genes were identified in budding yeast (DeFeo-Jones et al., 1983; Nakafuku et al., 1988), fission yeast (Fukui et al., 1986), the slime mould (Reymond et al., 1984) and *Drosophila* (Neuman-Silberberg, 1984). The RAS proteins in budding yeast were found to be functionally equivalent to the mammalian GTP-binding protein in being involved in the activation of adenylate cyclase (Toda et al., 1985) although not in response to growth hormones. The budding yeast contains two closely related but distinct genes; *RAS1* and

RAS2 that are characterized by highly conserved amino acid sequences that correlate with mammalian RAS proteins (Defeo- Jones et al., 1983; Dhar et al., 1984; Power et al., 1984). Although disruption of either the *RAS1* or the *RAS2* gene alone has no effect on growth of yeast, haploid spores containing disruptions of both *RAS* genes fail to germinate and grow, therefore at least one gene is essential. This defect can be complemented by mammalian *ras* genes (DeFeo-Jones et al., 1985; Kataoka et al., 1985). The *ras1.ras2* double mutant cells of yeast are unable to survive, like adenylate cyclase deficient mutant *cyr1*. The *bcy1* mutation of the protein kinase regulatory subunit can suppress lethality in *ras1.ras2* double mutant cells (Toda et al., 1985) presumably by allowing cAMP-independent protein kinase activity. Intracellular cAMP levels of *ras1* mutants are slightly low, while those of *ras2* mutants are markedly reduced compared to wild type cells. In the triple mutant *ras1.ras2.bcy1* cAMP levels are virtually undetectable (Matusmoto et al., 1985).

A *ras2* allele with a missense mutation designated $RAS2^{val19}$ confers capacity to activate adenylate cyclase without requiring GTP binding. $RAS2^{val19}$ causes growth and biochemical properties similar to the *bcy1* or *IAC* mutants (Kataoka et al., 1984). The similarity arises from elevated cAMP levels caused by $RAS2^{val19}$ which therefore resembles the *IAC* gene in its effect on cAMP level. Diploid cells containing $RAS2^{val19}$ showed poor sporulation like the *bcy1* mutant and failed to accumulate glycogen and trehalose. The $RAS2^{val19}$ mutation caused an increase in the levels of GTP-independent adenylate cyclase activity in the presence of *CYR1* product (Toda et al., 1985). Membranes from *ras1.ras2.bcy1* yeast cells lacked GTP-stimulated adenylate cyclase activity that is present in membranes from wild type cells, and membranes from $RAS2^{val19}$ strain had elevated levels of an GTP-independent adenylate cyclase activity. Mixing membranes from *ras1.ras2.bcy1* with membranes from *cyr1* which is deficient in catalytic subunit of adenylate cyclase restored GTP-dependent adenylate cyclase activity. These results suggest that the yeast RAS proteins are GTP binding proteins which modulate adenylate cyclase activity (Toda et al., 1985), and therefore indicate parallels with the stimulation in animal cells and suggest that cAMP is a highly conserved signal molecule that is regulated by conserved mechanisms. In yeast the control of RAS activity is not by growth hormone receptor as it is in animal cells, rather yeast cells respond to nutrient supply and some genes have been identified as important in modulating cAMP in response to nutritional status. There are the *CDC25* (unrelated to *cdc25* of *S. pombe*) and *IRA* genes which appear to control the RAS

GTP-GDP cycle (Broek et al., 1987; Daniel et al., 1987; Marshall et al., 1987; Robinson et al., 1987). The *CDC25* gene was originally identified as a temperature-sensitive class II start mutant (Hartwell, 1973). Mutants of *cdc25* are arrested in G1 phase and have low cAMP levels at the restrictive temperature. This decrease in cAMP depends directly on *CDC25* since it is suppressed when the cloned *CDC25* gene is reintroduced into the *cdc25* cells (Camonis et al., 1986). Furthermore, addition of exogenous cAMP causes a reversion of mutated phenotype (Martegani et al., 1986). These studies suggested that the *CDC25* gene product is involved in the regulation of cAMP metabolism and allows cell growth proliferation by interacting with the cAMP-mediated system. The *cdc25* ts mutants have been reported to have low levels of GTP- stimulated adenylate cyclase activity but the activity was restored upon addition of an exogenous GTP analogue (Broek et al., 1987; Marshall et al., 1987; Robinson et al., 1987; Daniel et al., 1987). Therefore it is the control of cyclase activity rather than the cyclase itself that is defected in *cdc25* mutant cells. The *CDC25* gene product may act in the RAS-adenylate cyclase pathway as an exchange factor to regenerate RAS-GTP from RAS-GDP. Evidence for this hypothesis is that only GTP-bound forms of RAS1 or RAS2 stimulate adenylate cyclase (Field et al., 1987, 1988) and the dominant mutation of *RAS2* which eliminates the GTPase activity of RAS (Robinson et al., 1987; Broek et al., 1987) and the *RAS2*^{val19} which has reduced-GTP affinity (Camonis and Jacquet, 1988; Power et al., 1989), is capable of bypassing the requirement for a functional *CDC25* gene product. However, other possibilities have been suggested, *CDC25* could serve a structural role by facilitating the association between RAS and adenylate cyclase, or could serve to direct adenylate cyclase or RAS to the proper subcellular location (Petitjean et al., 1990).

Another component of RAS activity control, which counterbalances the stimulatory effect of *CDC25* has been identified by mutation of the *IRA1* gene. It appears to act as an inhibitory regulator of the RAS-cAMP pathway (Tanaka et al., 1989). The *IRA1* gene product is required for maintaining low levels of cAMP when appropriate in yeast cells. Strains carrying *ira1* mutation have high cAMP levels and share some phenotype characteristics with the *bcy1* and *RAS2*^{val19} mutations, such as defects in sporulation and poor sensitivity to nitrogen starvation. Null alleles of *IRA1* can suppress the *cdc25-1* growth defect but not *ras1.ras2* or *CYR1* gene disruption. In the *cdc25.ira1* double mutation the two defects compensate each other and there is suppression of the growth

arrest of *cdc25* and of the heat sensitivity phenotype of *ira1*. The *ira1* phenotype can be suppressed by a *ras2* mutation but not a *ras1* mutation. From these results, it has been suggested that IRA1 function is antagonistic to CDC25 function possibly stimulating the conversion of RAS- GTP to RAS-GDP (Tanaka et al., 1989). The RAS1 protein function is perhaps regulated by the gene *IRA2* which has recently been identified as a homologue of *IRA1*, but this area is currently under investigation (reviewed by Gibbs and Marshall, 1989).

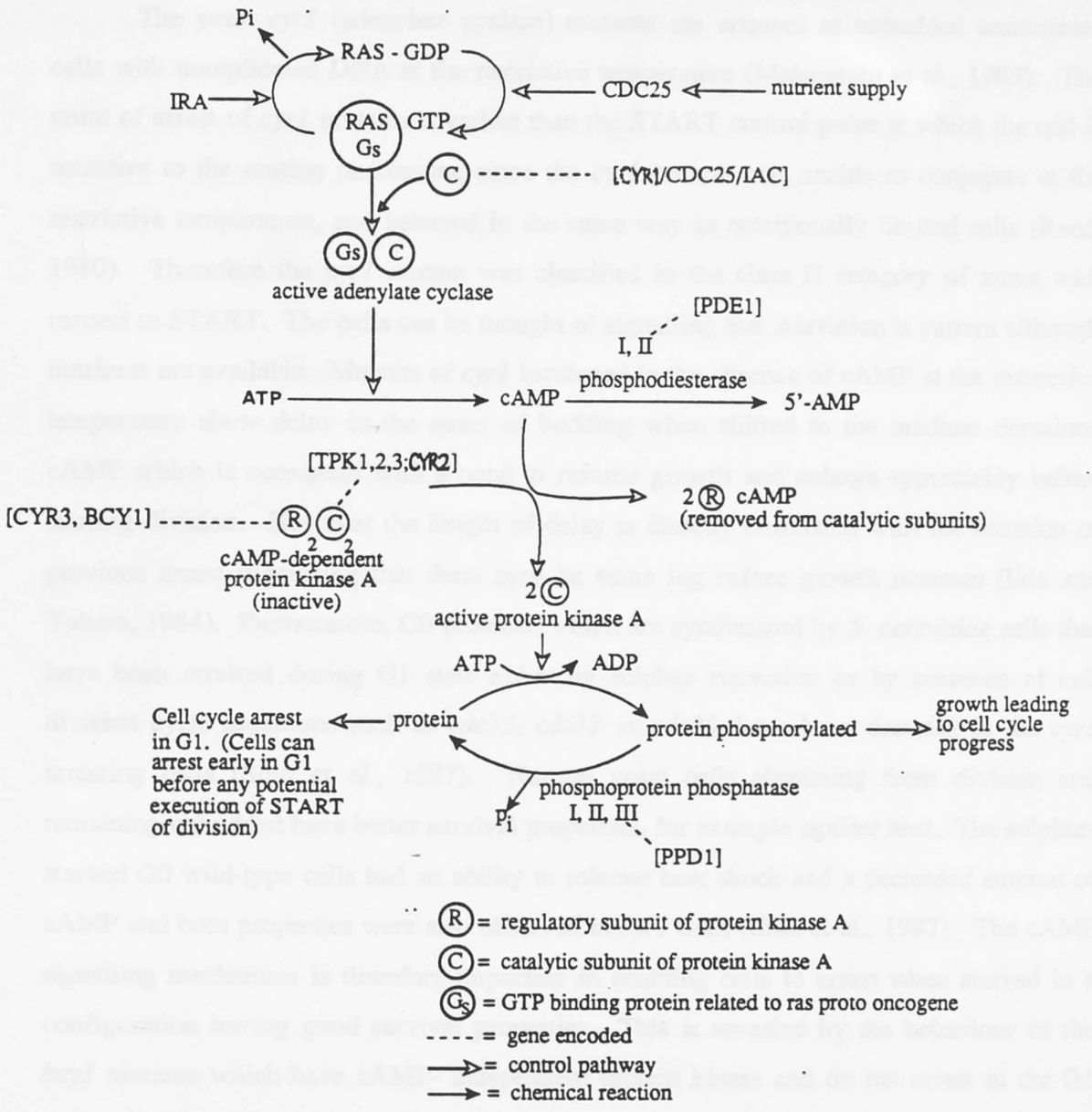
The present evidence concerning the cAMP pathway and its role in controlling growth and cell division in budding yeast is summarized in Fig. 1.2 and Table 1.1.

Fig. 1.2 Mechanisim by which cAMP acts as a signal for adequate nutrient supply and stimulates growth leading to division in budding yeast. The functions of the indicated genes products are discussed in the text and summarized in Table 1.1.

Table 1.1 Genes involved in the cAMP pathway

Nomenclature (alleles)	Gene product
RAS	GTP-binding regulatory protein
IRA	Enzymatic function unknown; probably involved in regulation of the RAS nucleotide state
CDC25	Enzymatic function unknown; probably involved in the RAS nucleotide state
CYR1(CDC35, IAC)	Adenylate cyclase
PDE1	Low-affinity (High Km) cAMP phosphodiesterase
CYR2, TPK1, TPK2, TPK3 (SRA3)	Catalytic subunits of the cAMP-dependent protein kinases
CYR3 (BCY1, SRA1)	Regulatory subunit of the cAMP-dependent protein kinase
PPD1	probably involved in regultion of phosphoprotein phosphatase

1.2


Pi
RAS - GDP
IRA
CDC25
nutrient supply
RAS
Gs
GTP
C
[CYR1/CDC25/IAC]
Gs
C
active adenylate cyclase
[PDE1]
I, II
phosphodiesterase
ATP
cAMP
5'-AMP
[TPK1,2,3;CYR2]
2 R cAMP
(removed from catalytic subunits)
[CYR3, BCY1]
R
C
2
2
cAMP-dependent
protein kinase A
(inactive)
2 C
active protein kinase A
ATP
ADP
Cell cycle arrest
in G1. (Cells can
arrest early in G1
before any potential
execution of START
of division)
protein
protein phosphorylated
growth leading
to cell cycle
progress
phosphoprotein phosphatase
I, II, III
Pi
[PPD1]
R = regulatory subunit of protein kinase A
C = catalytic subunit of protein kinase A
Gs = GTP binding protein related to ras proto oncogene
---- = gene encoded
= control pathway
= chemical reaction

Roles of cAMP in cellular function of budding yeast

The yeast *cyr1* (adenylate cyclase) mutants are arrested as unbudded uninucleate cells with unreplicated DNA at the restrictive temperature (Matsumoto et al., 1983). The point of arrest of *cyr1* mutants is earlier than the START control point at which the cell is sensitive to the mating pheromone since the *cyr1* mutant was unable to conjugate at the restrictive temperature, and behaved in the same way as nutritionally limited cells (Reed, 1980). Therefore the *cyr1* mutant was classified in the class II category of arrest with respect to START. The cells can be thought of signalling that starvation is current although nutrients are available. Mutants of *cyr1* incubated in the absence of cAMP at the restrictive temperature show delay in the onset of budding when shifted to the medium contained cAMP which is consistent with a need to resume growth and enlarge appreciably before starting division. However the length of delay is directly correlated with the duration of previous arrest suggesting that there may be some lag before growth resumes (Iida and Yahara, 1984). Furthermore, G0 proteins, which are synthesized by *S. cerevisiae* cells that have been arrested during G1 state either by sulphur starvation or by presence of cell division cycle mutations such as *cdc25*, *cdc33* or *cdc35*, have been detected in the *cyr1* arresting cells (Shin et al., 1987). Normal yeast cells abstaining from division and remaining unbudded have better survival properties, for example against heat. The sulphur-starved G0 wild-type cells had an ability to tolerate heat shock and a decreased amount of cAMP and both properties were also observed in *cyr1* cells (Shin et al., 1987). The cAMP signalling mechanism is therefore important in enabling cells to arrest when starved in a configuration having good survival properties. This is revealed by the behaviour of the *bcy1* mutants which have cAMP- independent protein kinase and do not arrest in the G1 state under condition such as starvation that arrests normal wild type cells in the G1 state (Matsumoto et al., 1983).

The process of meiosis leading to sexual reproduction which is a frequent response to low nutrient supply, by contrast with growth, requires low cAMP. The *cyr1*, *cyr2* and *CYR3* homozygous diploid cells which had low cAMP were capable of initiating meiosis and sporulating at the restrictive temperature even in rich media which are normally inhibitory to sexual reproduction, but in *bcy1*, *ppd1* and *RAS2*^{val19} homozygous diploid cells which had high cAMP and cAMP independent kinase activity meiosis could not be initiated and cells did not sporulate even in poor media (Toda et al., 1985; Reed et al., 1985;

Matsumoto et al., 1983). From these observations, it is concluded that cAMP works as a negative effector for sporulation and as a positive effector for growth. There is no evidence that cAMP interacts directly with START of division but adequate levels are a necessary precondition since cells must grow to a critical size to execute START (Pringle and Hartwell, 1981). Similarly only growing cells respond to mating pheromone and conjugate. Therefore cAMP can be seen as a positive effector for both division and conjugation.

In sporulation diploid yeast cells produce haploid nuclei by meiotic division and form ascospores. The *cyr1*, *cyr2* or *CYR3* homozygous diploid cells were able to complete meiotic division producing tetrad nuclei but there is some evidence of a positive contribution by cAMP to the meiotic process, once it has been initiated in the absence of high cAMP, because persisting low cAMP causes defects in meiosis in *cyr1*, *cyr2* or *CYR3* cells, in which only two nuclei are enclosed after meiosis. This is caused by defective spindle pole body forming during meiosis I (Uno et al., 1985). Spindle pole body formation is an early event in nuclear division (Hartwell, 1978) and the requirement for cAMP here may correlate with the apparent requirement for cAMP to complete START in the mitotic cycle which cycle which is soon followed by spindle pole body duplication.

Cyclic AMP in higher plants

At least some roles of cAMP in the control of metabolism and cell differentiation in mammals and in some microorganisms have been established but in higher plants the possible physiological roles of cAMP remain unclear.

The presence of cAMP has been detected in a variety of lower plants, for example, *Acetabularia* (Driessche et al., 1979; Minder et al., 1978), *Anabena* (Francko and Wetzel, 1980, 1981; Hood et al., 1979), *Chlamydomonas* (Bressan et al., 1980), *Chlorella* (Berchtold and Bachofen, 1977; Francko and Wetzel, 1980). Several investigations have demonstrated that most of the total cAMP found in these cultures had been excreted to the culture medium. The release of cAMP by these organisms is a poorly understood phenomenon.

In higher plants early attempts to demonstrate the existence of cAMP were not generally accepted. In these early investigations the method usually employed was isolation of a compound which either competed with radioactive cAMP for antibody binding or showed chromatographic properties similar to authentic cAMP. One such approach involved supply of [8- ^{14}C]adenine to germinating barley seeds (Pollard, 1970) or coleoptile (Solomon and Mascarenhas, 1971, 1972) and isolation of radioactive cAMP identified by chromatographic fractionation. These reports were criticized because the procedures may not have distinguished between adenosine 3':5'- cyclic monophosphate and its 2':3' isomer. Other methods of identification included enzymatic hydrolysis using cAMP-phosphodiesterase (Narayannan et al., 1970). This approach was criticized on the grounds that the result was valid only if the enzyme was absolutely specific for the substrate. Further attempts employed sequential chromatography by anion and cation exchange chromotography then thin layer chromatography (TLC) and high- voltage eletrophoresis for separation of cAMP (Brown and Newton, 1973). Although the purified compound was inseparable from a reference cAMP, there were counter-claims that unidentified adenine nucleotides, which might behave like cAMP in these systems, could exist in higher plants. Therefore the obstacle to cAMP identification when dealing with plant tissues was the possible presence of interfering substances that mimicked cAMP behaviour.

Some of the most compelling evidence for the existence of cAMP in plant tissues was derived by application of gas chromatography-mass spectrometry (GC-MC) analysis

(Johnson et al., 1980, 1981). Johnson and his co-workers demonstrated that samples from several plant tissues (*Nicotiana* callus, *Zea mays* kernels and *Phaseolus* seedlings) contained authentic cAMP which could be resolved from impurities by HPLC. Two impurities were found to be coumarin glucoside and scopolin, which was present in large amounts in tobacco callus (Johnson et al., 1980). Identification of cAMP in plants was further supported by Newton and co-workers who used mass spectrometry to demonstrate the occurrence of cAMP in *Phaseolus* tissues (Newton et al., 1980). These studies provided clear evidence for the existence of cAMP in the cells of higher plant cells. The extensive purification, especially using HPLC and GC-MS assay for cAMP, did not identify a physiological role of this molecule in higher plants. An experimental approach was difficult to identify because normal cell may have adequate cAMP and may not response to additional cAMP.

The presence of enzymes capable of cAMP metabolism was reported even before cAMP was definitely shown to occur in higher plants (reviewed by Francko, 1983; Newton and Brown, 1986). Adenylate cyclase activity had been reported in *Phaseolus* (Brown et al., 1977), *Pisum* (Hilton and Nesius, 1978), *Pinus* pollen (Katsumata et al., 1978) seedlings of *Trifolium* (Tu, 1978) and in some other plant sources (Brown and Newton, 1981). However, Hintermann and Parish (1979) found no evidence of adenylate cyclase in some plants such as *Bryophyllum, Euphorbia, Amaranthus, Trifolium, Zea* tissues, bean hypocotyl or onion meristem tissues. Negative data are difficult to evaluate because some tissues present difficulties for extraction of active enzyme or perhaps cAMP is produced in some higher plants by enzymes different from adenylate cyclase.

The other enzyme concerned with cAMP metabolism is phosphodiesterase. This enzyme has been reported in pea seedlings (Lieberman and Kunishi, 1969), tobacco pith (Wood et al., 1972), potato (Ashton and Polya, 1975; Shimoyama et al., 1972), barley (Vandepeute et al., 1973), carrot leaves (Venere, 1972), and several other plants (reviewed by Newton and Brown, 1986). Phosphodiesterase from some plants, such as pea seedlings (Lin and Varner, 1972), showed significant differences in properties from bacterial and mammalian counterparts. One difference was greater ability to hydrolyze 2':3'-cAMP rather than 3':5'-cAMP, resulting in production of 3'- AMP as the main hydrolysis product. The other difference was an acidic pH optimum. However, the properties of phosphodiesterase in *Phaseolus vulgaris* resembled that of the mammalian and yeast enzymes.

Phosphodiesterase of *Phaseolus vulgaris* is active towards 3':5'-cAMP but inactive towards 2':3'-cAMP and therefore produces 5'-AMP as the major end product of hydrolysis (Brown et al., 1975, 1977; Dupon et al., 1987). In the studies of the subcellular distribution of phosphodiesterases in *Spinacea* seedlings, chloroplast phosphodiesterase has more activity towards 3':5'-cyclic nucleotides than towards the 2':3'-isomers whereas the microsomal enzyme has greater acitivity towards 2':3'-cyclic nucleotides (Brown et al., 1979, 1980). Therefore the differences in results among the earlier findings could be caused by methods which resulted in selective extraction of a particular enzyme fraction. It has been reported that at least three types of phosphodiesterases can be present in plant tissues. For instance, three phosphodiesterases have been demonstrated in potato, one has greatest activity as a NAD pyrophosphatase, the second has activity towards 2':3'- cAMP as substrate and the third has activity towards 3':5'-cAMP as substrate (Ashton and Polya, 1975).

In animals and yeasts there is an interaction between calcium and cAMP controls since calcium activates cAMP breakdown by activation of phosphodiesterase via calmodulin. The discovery of calmodulins, which are known as activators of animal cyclic nucleotide phosphodiesterases, and other Ca^{2+}-binding proteins in plant tissues could lead to a better understanding of cAMP degradation in plants (Anderson et al., 1981; Grand et al., 1980; Lado et al., 1981; Bollig et al., 1978). The Ca^{2+}-calmodulin activation of plant phosphodiesterases has been noted in *Phaseolus* (Brown et al., 1979), *Spinacea* (Brown et al., 1980) and *Hordeum* (Grand et al., 1980). Although these data suggest many possibilities, there is still much to be learnt about the control of cAMP degradation in higher plant tissues. However as a point of similarity with mammalian and microbial cells it is established that among the varieties of phosphodiesterases present in higher plant tissues, at least one phosphodiesterases has a predominant specificity for 3':5'-cAMP. One possibility is that cAMP levels may be in part controlled in plants by regulation of phosphodiesterase as it is in mammalian and microbial systems.

Other components of the cAMP system identified in animal and fungal cells that have been reported in higher plants include cAMP-binding proteins which could control protein kinase activity. cAMP binding proteins activities have been found in tissues of *Phaseolus* (Brown et al., 1979), *Hordeum* (Brown et al., 1980) and wheat germ (Polya and Bowman, 1981). There is not yet published evidence for the presence of cAMP-dependent protein kinase in higher plant tissues. However, there are reports that cAMP promotes

protein phosphorylation in coconut milk (Janistyn, 1989) and specific inhibitors of protein kinase C inhibit bud initiation in *Torenia* stem segments, in which budding is induced by all agents that induced elevated cAMP (Ishioka and Tanimoto, 1990). Although the knowledge of protein kinases in higher plant tissues is still incomplete, it is possible that cAMP may exert some regulatory functions through cAMP-dependent protein kinases.

It is known that plant tissues contain all the essential components of cyclic nucleotide-mediated regulatory systems with the possible exception of cAMP dependent protein kinase. This poses the question as to whether a cyclic nucleotide mediated control mechanism is functional in plants as in animals and microorganisms. Approaches that have been adopted to evaluate the physiological roles of cAMP in plants were mostly influenced by the secondary messenger concept of cyclic nucleotide action in mammals. These strategies include observation of the effects of exogenously applied cAMP or its analogues, and of the effect of phytohormones on the synthesis of cAMP. Most of the investigations of phytohormone-cAMP relationships have centered around gibberellin (GA_3) and auxin (IAA). It has been reported that GA_3 stimulates the incorporation of [8-^{14}C] adenine into cAMP in barley (Pollard, 1970) and increases cAMP level in etiolated maize (Torantowicz-Marek and Kleckowski, 1975) and Jerusalem artichoke (Giannatasio and Macchia, 1973). There is some evidence to suggest that cAMP produces the same effects as GA_3. At the onset of germination, the activity of a number of hydrolytic enzymes such as ATPase, α-amylase or protease, can apparently be induced by either GA_3 or cAMP. However it is noticeable that this work has not been followed up at the molecular level in the last fifteen years. In addition, cAMP can also produce the same effect as GA_3 on growth response of a number of higher plants, such as lettuce, barley and soybean (reviewed by Newton and Brown, 1986).

IAA has also been reported to increase cAMP level in a number of plant tissues such as *Avena* (Salomon and Mascarenhas, 1972), soybean callus (Brewin and Northcote, 1973a) and *Cicer* (Azhar and Krishna, 1971). It is also reported that various physiological effects induced by IAA can also be elicited by cAMP. These effects are, for instance, enhancement of etiolated coleoptile elongation in corn and wheat, the promotion of growth in sunflower callus, oats and artichoke and a synergistic effect on differentiation of lettuce (reviewed by Newton and Brown, 1986). This evidence serves only to indicate that addition of

exogenous cyclic nucleotide may be capable of stimulating the effects of hormones but there is no evidence to establish that cAMP mediates the effects of these hormones.

The first indication that mutational analysis could be valuable in investigation of cAMP function has come from study of the haploid gametophyte of a moss. Cyclic AMP antagonism of the IAA-elicited response has been observed in the protonemal differentiation in moss *Funaria hydrometrica* (reviewed by Bhatla and Chopra, 1983). The first formed protenema is the chloronema which has transverse septae, round chloroplasts and hyaline walls. The chloronema gives rise to a caulonema which has oblique septae, fewer chloroplasts and brown walls. This differentiation is promoted by IAA. Supplementation of cAMP to a chloronema-repressed mutant, pg-I mutant which formed caulonema without the addition of IAA, enhanced the initiation of new chloronema filaments. Furthermore addition of phosphodiesterase inhibitors aminophylline, theophylline and I.C.I. 58,301 also enhanced the formation of new chloronema filaments in the IAA- treated wild-type culture (Handa and Jonri, 1976).

There is some evidence that cAMP has a role in plant cell proliferation since there are reports that the phosphodiesterase inhibitors, aminophylline and theophylline inhibit progress from G1 to S phase at germination of root meristem cells of *Haplopappus* embryos and the administration of cAMP in combination with aminophylline induced a similar effect in the same tissues although no check for altered cAMP level in treated cells was reported (Levi et al., 1981,1983). Therefore, these results possibly indicate involvement of cAMP in plant cell proliferation.

Cyclic AMP in *C. reinhardtii*

Amrhein and Filner have reported as early as 1973 the presence of a substance resembling cAMP in *C. reinhardtii*. This substance co-chromatographed with authentic cAMP, stimulated cAMP-dependent protein kinase from rabbit muscle and was destroyed by beef heart cyclic nucleotide phosphodiesterase. Later the existence of cAMP in *C. reinhardtii* was also reported by Bressan et al. (1980) and Sharaf and Rooney (1982 and 1985).

The physiological significance of cAMP in *C. reinhardtii* is mostly unknown. However Rubin and Filner (1973) found that cAMP production in *C. reinhardtii* was related to the flagellar function and regeneration. Increase in internal cAMP level by the addition

of PDE inhibitor, caffeine and aminophylline, inhibited flagellar movement and flagellar regeneration in wild-type cells of *C. reinhardtii*. cAMP has also been shown to have a stimulatory role during *C. reinhardtii* mating (Pasquale and Goodenough, 1987). The agglutination of gamete cells triggers an increase in cAMP level and addition of the cAMP analog, db-cAMP induces mating responses in non-mating gametes.

As in mammalian and other eukaryote cells, the level of cAMP in *Chlamydomonas* was found to fluctuate in synchronous cultures. The intracellular level of cAMP in *C. reinhardtii* was estimated at 25 pmol/g dry weight (or approximately 5-7.5 pmol/g wet weight) by Amrhein and Filner (1973) and 16 pmol/g wet weight by Bressan et al. (1980). Dissolved cAMP levels in culture filtrates were generally highest in stationary phase while most of the cAMP in actively growing cultures was associated with cells (Bressan et al., 1980). An intriguing thing about algal cAMP release is its magnitude. Several investigations have demonstrated that the majority of the total cAMP found in algal cultures often occurs in the extracellular dissolved form. The release of the cAMP to the culture is a poorly understood phenomenon. In synchronized cells rhythmic cAMP changes occurred. The level doubled during DNA replication and dropped as the replication ended (Sharaf and Rooney, 1985). Therefore it has been suggested that the surge of cAMP during S phase might trigger events of mitosis that follow. However these changes of cAMP level may be the response to the perturbation of the culture causes by synchronization procedure and not directly involved in the cell division since Sharaf and Rooney sampled during continuation of the synchronizing regime that involved changes in both temperature and illumination. Study of mutants that are specifically defective in cAMP pathways are therefore potentially very useful in providing direct information concerning possible essential contributions of cAMP to cell division in *Chlamydomonas*.

Enzyme activities present in *Chlamydomonas* indicate that the production and degradation mechanisms of cAMP are probably similar to those that are found in bacteria and animal cells; cAMP can be produced from ATP by adenylate cyclase (Hintermann and Parish, 1979; Bressan et al., 1980) and degraded by cyclic nucleotide phosphodiesterases (Fischer and Amrhein, 1974). The characteristics of cyclic nucleotide phosphodiesterases and adenylate cyclase of *Chlamydomonas* more closely resemble bacterial and animal enzymes than those found in higher plants (Fischer and Amrhein, 1974; Francko and Wetzel, 1981). *C. reinhardtii* phosphodiesterases exhibited an alkaline pH optimum, is

highly specific for 3',5'-cAMP, produces 5'-AMP as a major hydrolytic product and is inhibited by methylxanthine compounds, whereas phosphodiesterase enzymes from higher plants have acid pH optima, are insensitive to methylxanthine and the major product of their cAMP hydrolysis are 5'-AMP and 3'-AMP (review by Brown and Newton, 1981). Adenylate cyclase activity has previously been reported in whole cell extracts of *C. reinhardtii* vegetative cells (Hintermann and Parish, 1979) where its activity is similar to the adenylate cyclase that was found in slime mould. Adenylate cyclase has also been found in association with flagellar membrane of gamete cells and its properties resemble those of adenylate cyclase in ciliary membranes of the lower eukaryote *Trypanosoma cruzi* (Torruela et al., 1986), molluscs (Kopf and Vacquier, 1984) and mammalian sperm (Hindebrandt et al., 1985).

Clearly attempts to elucidate the physiological significant of cAMP in *C. reinhardtii* as well as in other photosynthetic organisms are still incomplete. One reason is the uncertainty of measuring cAMP and the difficulty of separating cAMP-induced effects from other metabolic variables. The isolation of cAMP-deficient mutants presented here could provide means to evaluate the significance of cAMP in this organism and by comparison possibly in higher plant cells.

Cell cycle control in *Chlamydomonas*

Chlamydomonas was selected for the present study because it is a member of the *Chlorophycae* which includes the higher nonvascular plants. It is unicellular and therefore permits the techniques of mutagenesis and selection within a large population that have been developed for microbial genetic studies. A further crucial advantage is that the extended vegetative phase of the life cycle is haploid therefore mutagenesis of the single gene copy will have an effect on the phenotype whereas in higher plants and animals that are diploid the presence of the other member of each gene pair can mask the mutant defect.

Until recently the cell cycle of *Chlamydomonas* has seemed perhaps fundamentally different from that of many eukaryotes since it often involves multiple fission to 2, 4, 8, or 16 daughter cells. However recent results in our laboratory have shown that the key control point of the *Chlamydomonas* cell cycle is commitment to cell number doubling that immediately precedes DNA replication leading to mitosis and cytokinesis. Functional equivalence can be recognized between commitment in *Chlamydomonas* and START of division in yeasts (John, 1984; Donnan et al., 1985) as will be emphasized below. The division cycle of *Chlamydomonas* differs from most eukaryotes simply in containing a timer period in G1 phase, which is therefore prolonged with the result that commitment and cell division occur at the approach of nightfall. At rapid growth rates the long G1 phase allows more than one doubling in cell mass, but size homeostasis is achieved by the successive recurrence of commitment, each to a cell number doubling and therefore leading to division numbers in the 2^n series. At very slow growth rates the cell cycle of *Chlamydomonas* is identical with that of other eukaryotes and probably with higher plants because G1 phase is then prolonged after expiry of the G1 precommitment timer until a minimum size for commitment has been attained. Division is then initiated only when the minimum size for commitment is reached and two daughter cells are formed as is common in other eukaryotes. It is uncertain in higher plants how important is the influence of cell size upon division. It seems very likely that cell size has an important influence since it has this function in the yeast and animal cells that have been studied as well as in the unicellular plant *Chlamydomonas*.

The cell cycle of *Chlamydomonas* growing at high growth rates has been illustrated previously (John, 1984 and McAteer et al., 1985) and is shown in Fig. 1.3.

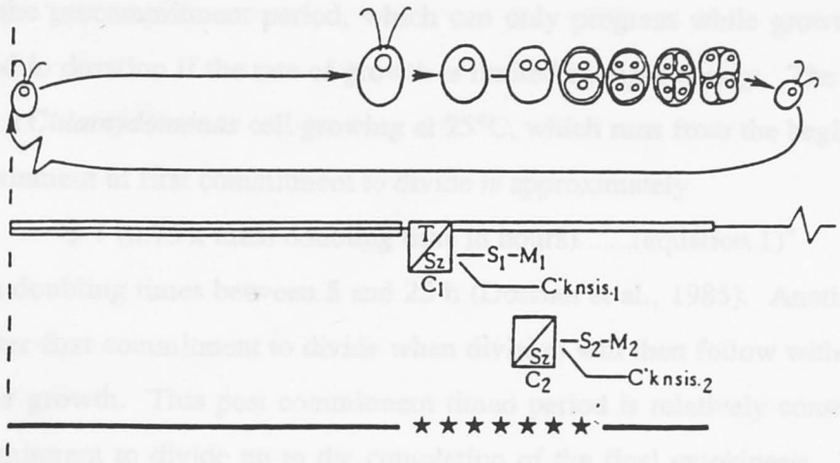


Fig. 1.3 Cell cycle of *Chlamydomonas* at high growth rate

C : commitment to divide

T/Sz : expiry of timer and fulfillment of cell size requirement.

– : time period in which no commitments to division can occur

★: time period in which commitment to division can recur if mother cell size is above critical minimum.

The attainment of commitment to divide occurs just prior to DNA synthesis. Each commitment resembles the START division control (John, 1984) in leading to a single round of DNA replication, mitosis and cytokinesis without requirement for further growth (Donnan and John, 1983). If the size of the mother cell is sufficiently large, further commitments can be attained and thus leads to the production of four, eight or sixteen daughter cells. The commitment process is terminated either by committed daughter cell mass falling below a critical value or by interruption of growth due to darkness. Cells which abstain from further commitments return to the precommitment state in which a timer period must be completed before another commitment is undertaken. Multiple fission is a consequence of the repetition of commitments to divide. These result in an

overlapping in time of individual sequences of DNA doubling, mitosis and cytokinesis (Donnan and John, 1984).

The overall timing of cell cycle duration that leads to a requirement for multiple fission at rapid growth rates results from operation of two different timers. One of them controls the precommitment period, which can only progress while growth occurs and is influenced in duration if the rate of growth is limited by light energy. The precommitment period in a *Chlamydomonas* cell growing at 25°C, which runs from the beginning of growth to the attainment of first commitment to divide is approximately

"5 + (0.75 x mass doubling time in hours).....(equation 1)"

at protein doubling times between 5 and 25 h (Donnan et al., 1985). Another timed period occurs after first commitment to divide when division will then follow without requirement for further growth. This post commitment timed period is relatively constant at 6 h from first commitment to divide up to the completion of the final cytokinesis. Total cell cycle duration, or mean generation time is then approximately:

"11 + (0.75 x mean doubling time in hours).....(equation 2)"

In *Chlamydomonas* the major rate-limiting event in progression to division is the attainment of commitment to divide that occurs just prior to S phase. There are similarities between this control point and the START control in yeasts (John, 1984). Both of them are located in late G1 just before S phase and commit a single doubling of DNA and cell number without further growth requirement (Donnan and John, 1984). They are recognized as the rate-limiting step because the initiation of DNA synthesis can be delayed if cells are growing slowly, and both controls require a critical minimum cell size. Once they have been executed, cells progress rapidly to S phase and division will follow even if growth is limited (Donnan and John, 1983).

The equivalence of commitment with START has been supported at the molecular level since the $p34^{cdc2}$ protein that regulates the start of progress to DNA replication and the initiation of mitosis (Nurse and Thuriaux, 1980; Nurse and Bisset, 1981) has been detected in *Chlamydomonas* (John, Sek and Lee, 1989). Participation of $p34^{cdc2}$ in the division cycle of *Chlamydomonas* is indicated by changes in amount and phosphorylation that coincide with the timing of control points in the cell cycle. Furthermore the biochemical identity of the division promoting enzyme (MPF) of a number of animal cells with $p34^{cdc2}$ described earlier indicates that there is a universality of division control at the molecular

level in eukaryotes. Recent results in our laboratory, showing that high level of a p34^{cdc2} - like protein occurs only in dividing higher plant cells, indicate that the plant kingdom probably shares at least some elements of the division controls in other eukaryotes.

This exciting convergence of molecular elements in division control which includes the plant kingdom suggests that control elements identified in *Chlamydomonas* may be relevant in other plants, as well as providing an important advance in understanding of division control in this widely studied model plant cell.

MATERIALS AND METHODS

SECTION II GENERAL MATERIALS AND METHODS

i. Materials

1. Chemicals

All biochemicals were supplied by the Sigma Chemical Company Ltd. General laboratory chemicals were supplied by Ajax Chemicals, Clyde Industries Ltd. Auburn Australia, Mallinckrodt Australia Pty.Ltd. Clayton, Victoria and BDH Chemicals (Australia) Pty.Ltd., Port Fairy, Victoria and were of analytical grade.

2. Equipments

Sterile plastic petri dishes (9 cm in diameter) were supplied by Johns, Australia. Autoclavable microbiological filters were purchased from Microflow Ltd. Fleet Mill, Minley Road, Fleet, Hampshire, GU13 8 RD. Sinterglass bubblers (Porosity "0") were obtained from Gallenkamp, Ledson Rd., Wythenshawe, Manchester, M23 9ND. Disposable syringe filter holders (0.2 μm) were obtained from NML, Sartorious, Germany .

ii. Methods

1. Description of the organism

Chlamydomonas reinhardtii is a unicellular green flagellate in the phylum Chlorophyta. The genus *Chlamydomonas* is within the family Chlamydomonadaceae. A schematic view of general features of a *Chlamydomonas* cell is shown in Fig. 2.1.1.

A *Chlamydomonas* cell is usually pear shaped with a pair of apical flagella. Cells of the *C. reinhardtii* strain have a diameter of the order 10 μm, however since the cell can grow up to sixteen fold in one cell cycle under rapid growth conditions the size of the given cell is greatly influenced by its time of observation in the cell cycle and by its previous division history (John, 1987). Large mother cell sizes are accommodated by division numbers in the series 2, 4, 8, 16; a form of division called multiple fission.

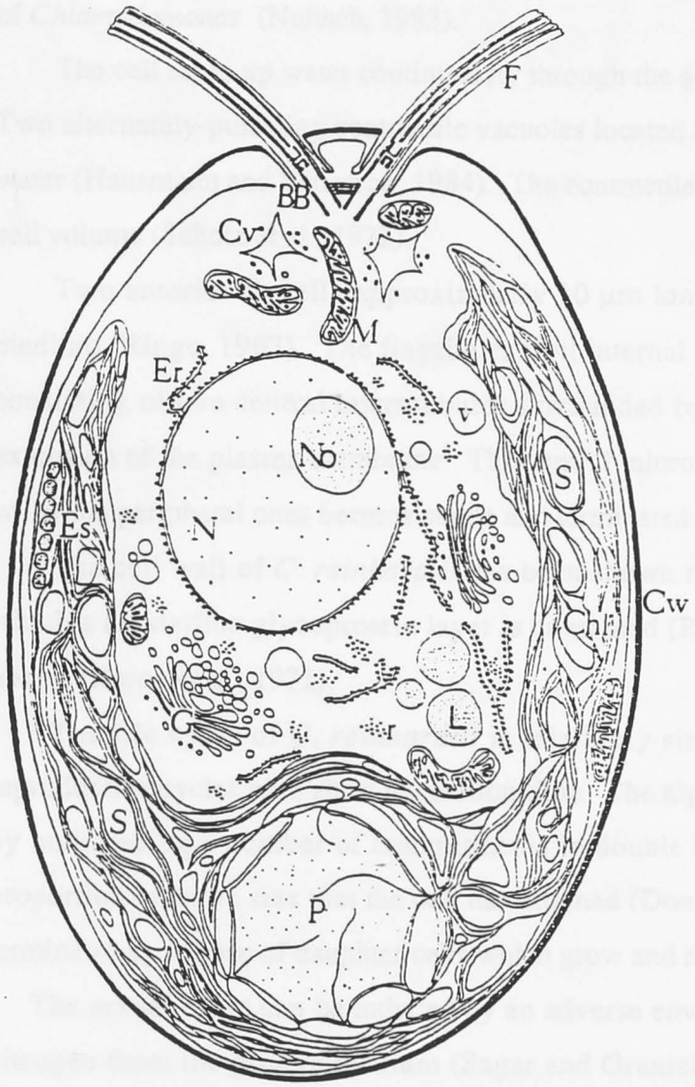


Fig. 2.1.1 A diagrammatic representation of a typical interphase cell of *C. reinhardtii*. Basal body (BB); Chloroplast (Chl); Contractile vacuole (CV); Cell wall (CW); endoplasmic recticulum (ER); Eye spot (Es); Flagella (F); Golgi body (G); Lipid body (L); Mitochondria (M); Nucleus (N); Nucleolus (No); pyrenoid (P); ribosome (r); starch grain (S); Vacuole (V) (Adapted from Etl;1976a and Harper, 1983 Ph.D thesis)

The *Chlamydomonas* cells usually contain a single nucleus and a single cup-shape chloroplast containing a pyrenoid. The nucleus occupies about 8% of the cell volume and in G1 phase its diameter is typically 2-4 μm depending upon cell size. The chloroplast occupies between one third to one half of the cell volume (Schotz et al., 1972). The cell possesses 10 to 15 highly branched mitochondria which occupy less than 5% of the cell volume and some are always sited near the flagellar bases (Bourque 1971).

The pyrenoid is one major site of starch formation (Griffiths, 1970) and its protein contains a high proportion of the CO_2 fixing enzyme ribulose 1,5-bisphosphate carboxylase (Hoober, 1984). On the outer surface of the chloroplast towards the cell wall

is a red-orange pigmented organelle called the eyespot or stigma. The eyespot is light sensitive, contains rhodopsin (Foster et al., 1984) and mediates the phototactic responses of *Chlamydomonas* (Nultsch, 1983).

The cell takes up water continuously through the semi-permeable plasmamembrane. Two alternately-pulsating contractile vacuoles located anteriorly expel the accumulating water (Hausmann and Patterson, 1984). The contractile vacuoles occupy about 8% of the cell volume (Schotz et al., 1972).

Two anterior flagella approximately 10 μm long propel the organism in liquid medium (Ringo, 1967). The flagella exhibit internal structure typical of an axoneme, consisting of two central microtubules surrounded by nine pairs, all within a tubular extension of the plasma membrane. The central microtubules extend to the flagella tip, while the peripheral ones become single and terminated some distance below it.

The cell wall of *C. reinhardtii* has been shown to consist of seven layers within which a crystalline glycoprotein layer is contained (Robert et al., 1972). Cellulose is absent (Horne et al., 1971).

The life cycle of *C. reinhardtii* is relatively simple. It has sexual and asexual reproductive cycles with short generation time. The alga normally reproduces asexually by undertaking a number of commitments to double DNA and cell number that is in proportion with the size that the cell has attained (Donnan et al., 1985). The cell cycle terminates by release of daughter cells which grow and repeat the cell cycle.

The sexual cycle can be induced by an adverse environment such as withdrawal of nitrogen from the growth medium (Sagar and Granick, 1954). A schematic diagram showing the sexual reproduction cycle of *C. reinhardtii* is shown in Fig. 2.1.2.

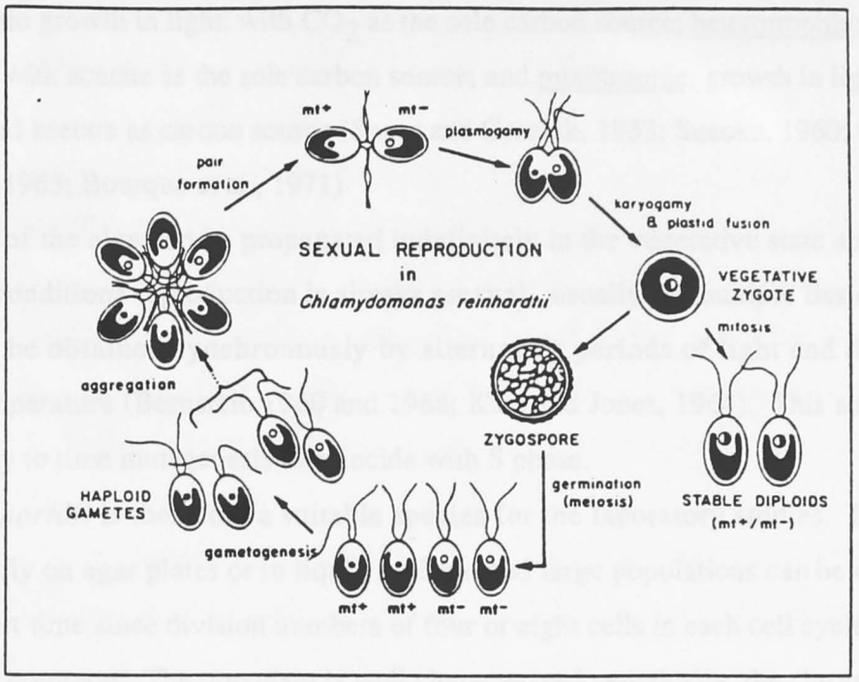


Fig. 2.1.2 Diagram of the sexual reproduction cycle in *C. reinhardtii*. The fusing of cells of different mating type, indicated as mt^+ and mt^-, produces a temporarily diploid zygote. The diploid zygote may develop into a resistant zygospore and on later germination it divides meiotically, forming four new haploid cells, which will probably divide repeatedly by mitosis. It was found that under normal laboratory conditions, 1-5% of the mated gamete pairs of *C. reinhardtii* do not form meiotic zygotes (zygospores); instead they divide mitotically shortly after mating as vegetative diploids and form discrete colonies within 3-4 days after plating of the mating mixture (Ebersold, 1967; reviewed by Harris, 1988).

The two isogamous sexes of *C. reinhardtii* designated mating type plus (mt^+) and mating type minus (mt^-), are permanently determined in a cell line by alternative alleles of a single nuclear gene (Smith and Regnery, 1950). When the complementary mating types are mixed, a number of compatible gametes become attached to each other. The gametic flagella of *C. reinhardtii* liberate a wall lysing enzyme (autolysin) and an adhesion factor, a glycoprotein agglutinin (Adair et al., 1982 and 1983). The gametes become naked through enzymatic dissolution of their wall prior to plasmogamy, which results in the formation of zygote. During a period of maturation the zygote forms a thick resistant wall and is called a zygospore. After maturation, a zygospore can be induced to germinate, undergoing meiosis and giving rise to four or eight daughter cells.

Wild type cells of *C. reinhardtii* can be easily grown in defined liquid medium or solid agar plates. Cells can be maintained under three conditions; phototrophic:

photosynthetic growth in light, with CO_2 as the sole carbon source; heterotrophic: growth in darkness, with acetate as the sole carbon source; and mixotrophic: growth in light, with both CO_2 and acetate as carbon source (Sagar and Granick, 1953; Sueoka, 1960; Gorman and Levine, 1965; Bourque et al., 1971)

Clones of the alga can be propagated indefinitely in the vegetative state and under favourable conditions reproduction is always asexual, usually by multiple fission. The culture can be obtained synchronously by alternating periods of light and dark at a constant temperature (Bernstein 1960 and 1968; Kate and Jones, 1964). This advantage makes it easy to time mutagenesis to coincide with S phase.

C. reinhartdii is therefore a suitable species for the laboratory studies. It can be cultured easily on agar plates or in liquid medium and large populations can be obtained within a short time since division numbers of four or eight cells in each cell cycle of 24 h duration are common. The organism is well characterized genetically, the chromosomes are defined, and genetic segregation follows the classical meiotic pattern (Levine and Ebersold, 1960; Levine, 1974), which makes it ideal for genetic studies.

2. Culture conditions

a. Culture of organism on agar plates

Cells were usually grown photoheterotrophically on TAPYPP agar plates (Table 2.2.1). Single colonies were picked up using a loop that had been sterilised by direct flaming and cooling and the cells were streaked onto a fresh plate. The plates were incubated under fluorescent lights giving 200 $\mu Em^{-2}s^{-1}$ of PAR in the range 400-700 nm for 7 days at 21°C then transferred to dim light. Cells were subcultured monthly onto fresh TAPYPP agar plates.

Reserve cultures were maintained as stabs in 2 ml TAPYPP agar in sterile, glass vials. Single colonies were stabbed directly into TAPYPP agar and the vials were placed in the light at 21°C for 2-3 days then transferred to dim light.

The phenotypes of the mutants were checked for temperature-sensitivity and caffeine resistance at each subculture. Temperature-sensitivity was checked by streaking cells very lightly onto TAPYPP plate and incubating at 33°C. Caffeine resistance was tested by streaking cells on 3 mM caffeine plates (Methods 5.c.) and incubated at 21°C. After 24 to 48 h, plates incubated at 33°C were examined for colonies with non-dividing large cells indicative of division block. After 7 to 10 days, the corresponding colonies were examined for their ability to grow on the caffeine containing agar plates. At least cells from 8 single colonies of each mutant were tested for temperature-sensitivity and caffeine resistance on plate. The colonies that grew on caffeine plates at 21°C and gave non-dividing large cells at 33°C were selected for propagation.

b. Culture of organism in liquid medium

Liquid cultures of cells were normally grown in TAPYPP medium in sterile 50-250 ml Erlenmeyer flasks fitted with cotton wool and muslin tops that were each held in place with a rubber band. Cells were picked from a single colony on an agar plate and were resuspended into the medium. The flasks were shaken on an orbital shaker under fluorescent light giving 200 $\mu Em^{-2}s^{-1}$ of PAR in the range 400-700 nm at 21°C. The cell cultures were left shaking until cell number reached exponential phase (1-5 x 10^6 cells/ml).

c. Synchronizing procedure

Prior to synchronization, cells were grown in a small volume of TAP medium which was prepared as described in Table 2.2.1, then transferred to a bigger volume e.g. 1-2 l of TAP medium in a 2.5 l Roux bottle (70 x 150 x 300 mm). The cultures were continuously aerated with air enriched with 0.5% (v/v) CO_2. The air mixture was sterilised by passing it through a "microflow" miniature filter and humidified by passing through sterile distilled water. The humidified air was delivered through a glass sinter (Porosity"0") at the rate of 1 l of air per min and 10 ml per min CO_2 per 2 l culture. The culture bottles were illuminated on both sides of the bottles by warm white fluorescent lights giving 200 $\mu Em^{-2}s^{-1}$ of PAR in the range 400-700 nm. The cultures were maintained at a constant temperature of 21°C.

Synchronization was achieved by alternating periods of 14 h illumination and 10 h darkness, L/D (Lorenzen, 1970). Cell cultures were maintained in synchrony by regular dilution to the density of 1.0 x 10^6 cell/ml at the end of each dark phase. To monitor synchrony cell numbers and cell size were measured with a Coulter Counter as described in Methods 3.b. At least 5 L/D cycles were employed to achieve a full synchrony. The normal profile of synchronously formed daughter cells is shown in Fig. 2.2.1.

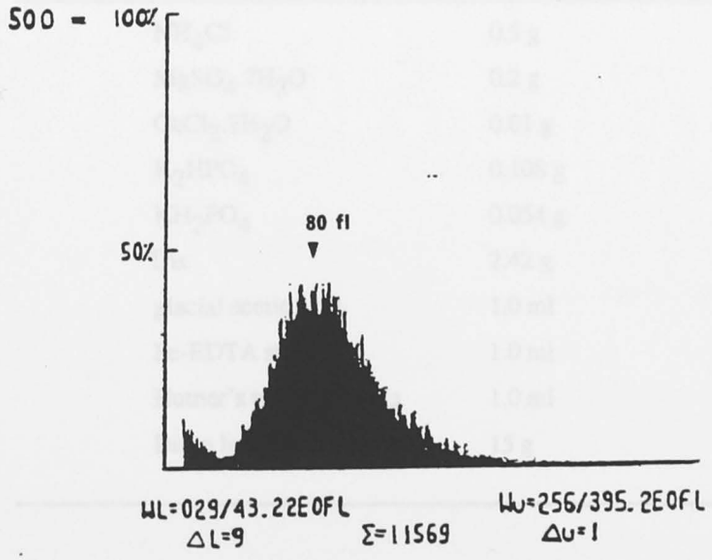


Fig. 2.2.1 Profile illustrating a uniform population of *C. reinhardtii* after a successful synchronization. Cells were grown in TAP medium with alternative 14:10 h, light:dark periods and continuously aerated with humidified air enriched with 5% CO_2. The culture was regularly diluted at the end of each dark period to 1 x 10^6 cells/ml. The mean cell volume (MCV) of vegetative cells at interphase was approximately 80 fl.

d. Long term storage of the organism

Cells can be kept viable for years in liquid nitrogen. Cells to be frozen were grown in LSHSMAYPP medium (Table 2.2.4) until cell number reached late exponential density (5-6 x 10^6 cells/ml). Aliquots of 0.3 ml of cells were added to solutions containing dimethylsulfoxide (DMSO), which were filtered-sterilised, to obtain the final concentration of 6%, 8% and 10% (v/v) DMSO in a final volume of 0.5 ml in 2 ml sterile-storage ampule (Flow Laboratories, U.K.). Triplicate sets of these treatments were needed for each mutant. After 15 min incubation under light at 21°C on a shaker, the tubes were placed in a polystyrene container so that cells froze slowly at -70°C in a freezer. After 1-7 days at -70°C one tube of each DMSO concentation was thawed quickly at 37°C. Aliquots of 0.2 ml were plated out on TAPYPP agar plates and incubated at 21°C under light for 10 days. Colonies were counted for detection of viability and replicates of samples with good viability were transferred to long term liquid nitrogen storage.

e. Composition of culture media

All media were made up in double glass distilled water.

Tris-acetate phosphate (TAP) medium used as liquid and also solidified as agar plates, is based on the medium of Gorman and Levine (1965) and modified according to Lien and Knutsen (1976) by supplying iron as an Fe-EDTA complex with Na_2EDTA.

Table 2.2.1 TAP medium

Compound	per litre	Final concentration mM
NH_4Cl	0.5 g	9.35
$MgSO_4.7H_2O$	0.2 g	0.81
$CaCl_2.2H_2O$	0.01 g	0.13
K_2HPO_4	0.108 g	0.63
KH_2PO_4	0.054 g	0.39
Tris	2.42 g	20
glacial acetic acid	1.0 ml	17
Fe-EDTA stock	1.0 ml	see Table 2.2.3
Hutner's trace elements	1.0 ml	see Table 2.2.2
Difco bacto agar	15 g	1.5%

TAPYPP medium, or agar plate was made up as described in Table 2.2.1 with the addition of 0.4 g of yeast extract and 0.4 g of proteose peptone per 1 l to provide a richer nutrient medium.

Table 2.2.2 Hutner's trace element

Compound	per liter	Final concentration mM
H_3BO_3	11.3 g	185
$ZnSO_4.7H_2O$	8.8 g	31
$FeCl_3.6H_2O$	4.8 g	18
$MnSO_4.4H_2O$	1.6 g	7.2
$Na_2MoO_4.2H_2O$	1.0 g	4.1
$CuSO_4.5H_2O$	0.8 g	3.2
$CoCl_2.6H_2O$	0.2 g	0.8
$Na_2EDTA.2H_2O$	20.0 g	54

Table 2.2.3 Fe-EDTA solution

Compound	per liter	Final concentration mM
$FeSO_4.7H_2O$	6.9 g	25
Na_2EDTA	9.3 g	25

Low sulphur high salt acetate (LSHSMA) medium was a modified high salt medium (HSM) of Sueoka et al. (1967); containing reduced sulphate to suit the growth of wild-type and with the addition of Mg^{2+} and acetate. Trace elements were supplied at the concentration proposed by Kuhl and Lorenzen (1964).

Table 2.2.4 LSHSMA medium

Compound	per liter	Final concentration mM
NH_4Cl	0.5 g	9.535
$MgSO_4.7H_2O$	0.2 g	0.81
$MgCl_2.6H_2O$	0.15	0.74
$CaCl_2.2H_2O$	0.01 g	0.13
K_2HPO_4	1.44 g	8.29
KH_2PO_4	0.72 g	5.18
Fe-EDTA	1 ml	see Table 2.2.3
trace element	1 ml	see Table 2.2.5

Table 2.2.5 trace element

Compound	per liter	Final concentration mM
H_3BO_3	0.061 g	1.0
$MnSO_4.4H_2O$	0.223 g	1.0
$ZnSO_4.7H_2O$	0.287 g	1.0
$CuSO_4.5H_2O$	0.0025 g	10 μM
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0124 g	10 μM
conc. H_2SO_4	1 ml	18.7

LSHSMAYPP medium was prepared as described in Table 2.2.4 with addition of 0.4 g yeast extract and 0.4 g of proteose peptone per 1 l.

Maturation medium 12 mM acetate (-NHSMA) This medium is based on VanWinkle-Swift (1977). The lack of nitrogen in this medium makes it suitable for the induction of gametes.

Table 2.2.6 -NHSMA medium

Compound	per liter	Final concentration mM
Na acetate	1.15 g	12
$MgSO_4.7H_2O$	0.02 g	0.081
$CaCl_2.2H_2O$	0.01 g	0.13
K_2HPO_4	1.44 g	8.29
KH_2PO_4	0.72 g	5.18
Fe-EDTA	1 ml	see Table 2.2.3
trace element	1 ml	see Table 2.2.5
Difco Bacto agar	40 g	4%

3. Cell number estimation

a. Estimation by haemocytometer

A sample of 1 ml culture was fixed by addition of 6 µl of 35% formaldehyde to a final concentration of 0.20% (v/v) formaldehyde. Aliquots of 10 µl of the fixed sample were placed into each haemocytometer chamber (Newbauer, Brand, Germany). Cells were then counted by microscopic inspection. The average cell density was estimated from a count of at least 500 cells.

b. Estimation of cell number and cell volume by Coulter Counter

Cell number and cell size were measured with a Coulter Counter Model ZB (Coulter electronics, Pty., Ltd., NSW 2100, Australia) which was connected to a Coulter Channelyzer C-250 and a printer (EPSON FX-800). The system was equipped with a 80 µm orifice (Coulter electronics Ltd., Northwell Dr., Luton Beds, England) and calibrated with latex particles of 15 µm in diameter. 0.8% NaCl solution was used as counting electrolyte.

The sample to be counted was fixed in formaldehyde at the final concentration of 1% formaldehyde and was diluted to less than 50,000 cells/ml with the counting electrolyte before estimation in the Coulter Counter. Each sample was counted four times and the average cell number was calculated. Mean cell volume (MCV) was read from the channelyzer display by moving a cursor, which indicated a lower cell volume threshold below which cells were not scored, until the total cell count was divided equally (scored number equal to half total number) and the cell volume identified by that threshold was recorded as a MCV.

4. Mithramycin staining of DNA

For inspection of nuclear division state to determine phase of the cell cycle, nuclear DNA was observed by staining cells with mithramycin.

a. Preparation of cell samples for mithramycin staining of DNA

Aliquots of 1 ml of cell sample in eppendorf tubes were centrifuged at 5000 rpm for 1 min and pelleted cells were resuspended in 150 µl of the same medium. To dehydrate the cells, a series of ethanol additions each followed by 20 s of mixing were made

as follows: 150 µl of 50% ethanol, 150 µl of 100% ethanol, then 675 µl of 100% ethanol. The sample, now in 80% ethanol, could be stored at -20°C after this step. For chlorophyll extraction, cells in 80% ethanol were further treated by placing in a 50°C water bath for 5 min with occasional shaking. Cells were again centrifuged and the pellets were resuspended in 300 µl of 80% ethanol. The sample could be extracted again if not fully decolorised or could be stored at -20°C after this step. For rehydration, 180 µl of distilled water was added to the chlorophyll extracted cells in 300 µl 80% ethanol mixed, and followed by a further 480 µl of distilled water and mixing. Cells were centrifuged at 3000 rpm for 1 min and the pellet was resuspended in 50 µl of mithramycin (5 µg/ml).

b. Mithramycin staining solution

The mithramycin staining solution (5 µg/ml) was prepared by dissolving 0.1 mg of mithramycin (Sigma) in a solution containing 30 mM $MgCl_2$, 1 mM EDTA, 5mM Tris-HCl pH 7.4 and 50% ethanol (v/v). This solution was kept in darkness at -20°C.

c. Fluorescence microscopy

For fluorescence observation, a volume of 5 µl was taken from the suspension containing cells that were stained in mithramycin (from a., above) and spread on a clean slide. The cells were dried briefly at room temperature, mounted with antifading solution (AF1; City University, London), covered with coverslip and sealed with nail vanish. Cells were examined using a Zeiss photomicroscope III equipped with epifluorescence illumination, standard UV, rhodamine, FITC filter sets and a Neofluor phase 3 (100 x 1.25) oil immersion objective. For observation of mithramycin fluorescence a FITC filter was used.

d. Photography

Photographs were taken on Kodak Tmax 400 film which was pushed to 1600 ASA by developing in Kodak TMax developer (Kodak, N.Y.,USA). Automatic exposure times were monitored throughout and was usually about 15-20 s. All cells were printed here at the same magnification so that relative sizes of cells can be judged directly.

5. Isolation of temperature-sensitive cAMP-requiring cell division cycle mutants

Mutations were induced by treating wild-type cells with ethyl methanesulphonate (EMS; purchased from Sigma) and screening for the colonies that grew on 3 mM caffeine plates at 21°C but were blocked in division at 33°C on TAPYPP agar plates. The selected temperature-sensitive caffeine resistant colonies were then further tested for cell division block in liquid medium. Then the putative temperature-sensitive colonies were tested for cAMP or dibutyryl cAMP requirement for cell division in liquid medium at 33°C.

a. EMS dose survival curve

To determine an effective dose of EMS for mutagenesis, cell survival after exposure to varying concentrations of EMS was studied. Wild- type strain (cc-125^{+}) was grown asynchronously in LSHSMA medium at 21°C. Cells were harvested by centrifugation at 5000 rpm for 5 min, washed twice with LSHSMA medium then resuspended at a higher cell density so that they could be mixed with EMS and attain the original cell density. A 400 mM stock solution of EMS in LSHSMA medium was made up freshly and filtered- sterilised before use. The stock EMS solution was added to sterile universal bottles to make final concentrations when eventually mixed with cells of 0, 40, 60, 100, 140 and 180 mM of EMS. The cell suspension was then added to each bottle to give a final density of 1 x 10^{6} cells/ml.

Cells in EMS were shaken for an hour under light at 21°C, then washed twice with LSHSMA medium. The mutagenized cells were resuspended in LSHSMAYPP medium at 3 - 5 fold dilution. The cells were allowed to grow in shaken culture for 18-24 hours at 21°C, after which the cells were diluted to 2500 cells/ml for plating. A 200 µl aliquot of each culture was plated on TAPYPP agar plates. After 10 days incubation at 21°C, surviving colonies were counted. The concentration of EMS giving 20% survival was identified. This concentration varied with age and batch of mutagen. A typical curve is shown in Fig. 2.5.1.

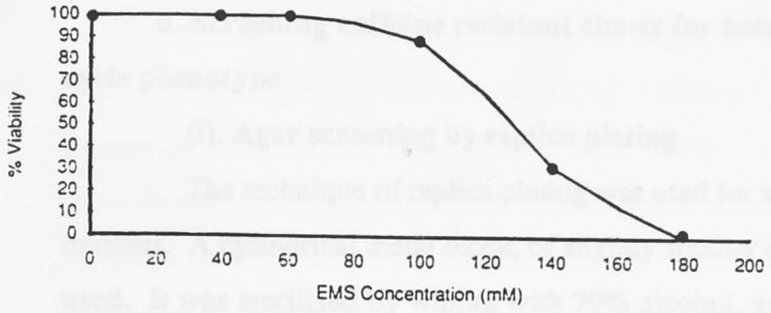


Fig. 2.5.1 EMS doses survival curve. Wild-type cells of *C. reinhardtii* were treated with EMS concentration range from 40 mM to 180 mM. The treated cells were diluted to 2500 cells/ ml and 0.2 ml were plated on TAPYPP plates and the colonies forming per plate were counted. Recovery of cells treated with 40-100 mM EMS was 90-100%. Recovery of cells treated with 140 mM EMS was 30% and at 180 mM EMS recovery was 1%.

b. Induction of mutants in synchronous culture

Cells of wild-type (cc-125^{+}) were grown synchronously, as described earlier in Methods 2.c, in LSHSMA medium. After five cycles of synchronous division cells were harvested at 12.5 h of the cycle (during the light period). Pellets were resuspended in LSHSMA medium and mixed with stock filter-sterilised of 400 mM EMS solution added to the final concentration of 0, 100 and 140 mM EMS at 1 x 10^6 cells/ml. Cells were shaken under continuous light at 21°C for 1 h, then washed twice with LSHSMA medium and diluted to 3-5 times lower than the original cell density with LSHSMAYPP. After 18 to 24 h of shaking at 21°C, a portion of cells was diluted to 2500 cells/ml for plating on TAPYPP plates, and the undiluted culture was plated on 3 mM caffeine plates was described in Methods 5.c.

c. Isolation of caffeine-resistant mutants

Plates containing 3 mM caffeine were prepared by adding 50 ml of stock 120 mM caffeine solution, prepared in TAP medium and filtered sterilised before use, to 2 l TAP agar medium. Aliquots of 0.2 ml of cell suspension at 1x10^6 cells/ml was pipetted directly onto the surface of an agar plate and evenly spread with a bent glass rod, which was sterilised by dipping into alcohol that was burnt off. After the spread cell suspension

had dried onto the surface of the agar, the plates were inverted and incubated under light at 21°C until colonies were visible which normally took about 10-14 days. Each plate carried $2x10^5$ cells but the majority of these were sensitive to caffeine.

To determine viable cell density after mutagenesis, 500 cells were spread on control plates without inhibitor, by diluting the cell suspension to 2500 cells/ml and plating out 0.2 ml.

d. Screening caffeine resistant clones for temperature-sensitive cell division cycle phenotype

(i). Agar screening by replica plating

The technique of replica plating was used for screening of temperature-sensitive mutants. A cylindrical metal block, of slightly smaller diameter than the agar plates, was used. It was sterilised by wiping with 70% alcohol, and then covered at the upper end with a piece of sterilised velvet. The velvet was held in place with a perspex ring of diameter slightly bigger than the metal block pushed down over the velvet holding it flat. The velvet was pressed firmly onto the surface of agar plates on which normal caffeine sensitive cells had died and the minority of caffeine resistant cells induced by mutagenesis had formed colonies. The inoculated velvet was then pressed onto fresh agar plates. All plates were marked with a specific reference point so that the colonies on the replica plates could be compared with the corresponding colonies on the master plate. The replica plates containing 3 mM caffeine were incubated at 21°C and the corresponding plate containing no selective agents were incubated at 33°C. Each pair of replica plates was screened to detect colonies that were caffeine resistant but unable to proliferate at 33°C.

After initial screening, each temperature-sensitive colony was picked off the 21°C plate and retested by streaking onto a caffeine plate that was incubated at 21°C and an unsupplemented plate that was incubated at 33°C. After 24 and 48 h, plates at 33°C were inspected microscopically for cells that failed to divide but had increased in size. These colonies were further screened in liquid medium.

(ii). Liquid medium screening for cdc arresting caffeine resistant mutant

Each putative temperature-sensitive cell division cycle caffeine-resistant mutant was further screening in liquid medium. A loopful of cells were inoculated in TAPYPP medium and grown at 21°C until the culture reached exponential phase. Cell suspensions

were then dispensed equally into 2 sterilised tubes containing fresh TAPYPP medium. The initial cell density of each tube was 0.2 x 10^6 cells/ml. One of them was incubated at 21°C whereas the other was incubated at 33°C. After 24 and 48 h 1 ml aliquots of each sample were fixed with 0.2% final concentration of formaldehyde (Methods 3.a) cell numbers and cell sizes were observed microscopically. Cells which were unable to divide but had increased in size were selected for further investigation.

e. Test for cAMP requirement for division at 33°C in caffeine resistant mutant

Each temperature-sensitive caffeine resistant strain was grown in TAPYPP medium at 21°C. During exponential growth the cell suspension was distributed into a set of sterilised 30 ml universal bottles. The compositions of this testing set were prepared as indicated in the following table. Each culture was of 10 ml volume and had an initial density of 2 x 10^5 cells/ml.

Table 2.5.1 Medium set to test for cAMP/db-cAMP requirement

Tube No	Treatment	Incubation °C	Final concentation mM
1	control	21	-
2	control	33	-
3	cAMP	33	1
4	cAMP	33	5
5	5'-AMP	33	1
6	5'-AMP	33	5
7	db-cAMP	33	0.5
8	db-cAMP	33	1
9	butyrate	33	0.5
10	butyrate	33	1

Adenosine 3',5'-cyclic monophosphate (cAMP), Adenosine 5'-monophosphate (5'-AMP), N^6-2'-O-dibutyladenosine 3',5'-cyclic monophosphate (db-cAMP) and n-butyric acid were purchased from Sigma in the form of sodium salts.

The testing set of cultures was incubated at the temperatures indicated with continuous shaking and illumination. At 24 h intervals 1 ml aliquots of cell suspension

were removed and fixed according to Methods 3.a and 4.a for the determination of cell number and for fluorescence microscopy. The response of each treatment was quantified in terms of cell number.

6. DNA Estimation

Two different types of method were employed. The first type of method was biochemical and employed two different chemical reactions; diphenylamine and diaminobenzoic acid dihydrochloride (DABA.2HCl) to measure total DNA. The second type of method measured DNA specifically in the nucleus using quantitative microspectrofluorometry.

a. Chemical assays

(i) Preparation of extracted cells for diphenylamine and DABA.2HCl assays

Duplicate samples were taken from synchronous cultures grown at 21°C under periodic illumination, or from asychronous cultures that were grown at 21°C or 33°C with continuous shaking and illumination. A number of cells between $50x10^6$ and $150x10^6$ was harvested by centrifugation at 5000 rpm at 4°C for 10 min. The supernatant was discarded and packed cells were assayed directly or kept frozen at -80°C.

The extraction procedure was modified from the method of Hopkins et al. (1972) and Lien and Knutsen (1976a). Samples were thawed quickly at 37°C and 1 N NaOH was added immediately. The cell suspensions in NaOH were allowed to stand for at least 3 h at room temperature, then were neutralized with an equal volume of cold 1 N HCl. Cells were then spun down at 3000 rpm for 5 min and the supernatant was discarded. Cold 50% (w/v) trichloroacetic acid (TCA) was added to the pellets then left on ice for 30 min. After centrifugation at 3000 rpm for 5 min, pellets were washed twice with cold 10% TCA and then twice with ethanol:ether (3:1) at room temperature. Nucleic acid was then extracted by suspending the pretreated cells in 0.5 N perchloric acid (PCA) and incubating the suspension at 90°C for 20 min. Standards described below (ii) were hydrolysed in parallel. The suspension was stirred frequently during the extraction procedures. Insoluble cellular materials were removed by centrifugation. Supernatants were used as the nucleic acid source in the assay procedure.

(ii) Reagents and standards for diphenylamine and DABA.2HCl assay

1. Diaminobenzoic acid dihydrochloride, DABA.2HCl (Sigma) was dissolved in distilled water to the final concentration of 20% (w/v) immediately before use.

2. Burton's reagent was prepared by dissolving 1.5 g diphenylamine in 100 ml glacial acetic acid. The reagent was stored in darkness at 4°C. Before use 0.5 ml of diluted acetaldehyde, which was a mixture of 1 ml cold acetaldehyde and 49 ml distilled water, was added to 49 ml of diphenylamine-acetic acid solution.

3. Standard DNA solutions were prepared by dissolving salmon sperm DNA (Pharmacia) in 5 mM NaOH to the concentration of 1 mg/ml. The stock solution was diluted to 100, 200, 400, 600, 800 µg/ml in 5 mM NaOH and stored frozen. Working standards were prepared by pipetting 0.1 ml of each stock solutions into 1.9 ml of 0.5 N PCA. The standards therefore contained 10 to 80 µg DNA. A blank was prepared by adding 0.1 ml of 5 mM NaOH to 1.9 ml of 0.5 N PCA. Standards were then hydrolyzed together with unknown samples at 90°C for 20 min.

(iii) Reaction mixtures and measurements

The diphenylamine assay method was based on that of Hopkins et al. (1972). 1 ml of acid extracted sample was mixed with 2 ml Burton's reagent. The mixtures were allowed to stand at 30°C for 14-20 h. Absorbances were measured at 600 nm.

The DABA.2HCl assay method was based on that of Lien and Knutsen (1976a). 0.2 ml of acid extracted sample was mixed with 0.3 ml of 1 N NaOH, and 0.2 ml of 20% (w/w) DABA.2HCl. The mixture was incubated at 60°C for 40 min. Then the reaction was stopped by adding 4 ml of 0.6 N PCA. Fluorescence was measured by using a fluorescence spectrophotometer Hitachi F.3000 (Hitachi, Ltd. Tokyo, Japan). Maximum emission intensity was obtained by using an excitation wavelength at 430 nm and emitted light was measured using a 530 nm interference filter for selecting only the emission light.

Using Salmon sperm DNA as a standard, linear standard curves were obtained from both reactions (Figs. 2.6.1a and b). With the DABA.2HCl method, standard DNA preparations of Salmon sperm DNA treated with PCA yielded an identical excitation-emission pattern as the DNA extracted from *Chlamydomonas,* as shown in Fig. 2.6.1c.

Fig. 2.6.1 Salmon sperm DNA standard assayed by

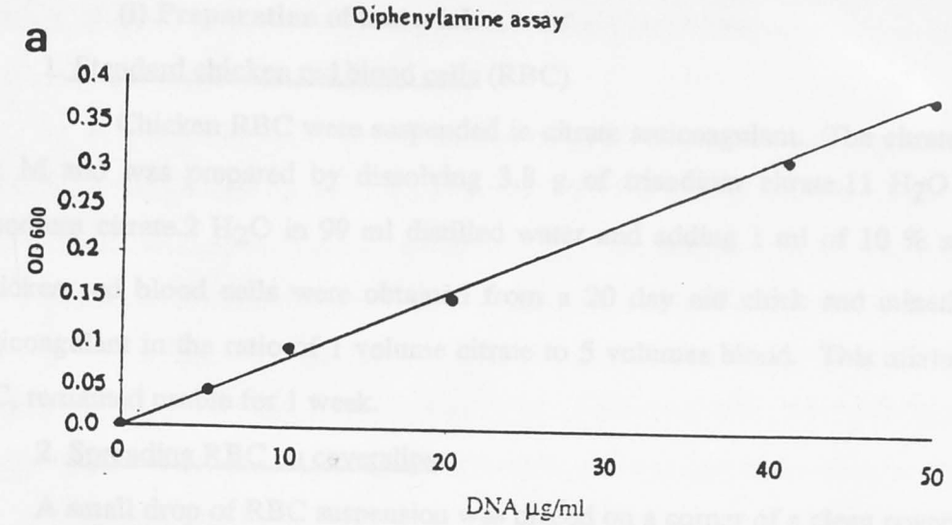
a. diphenylamine reaction

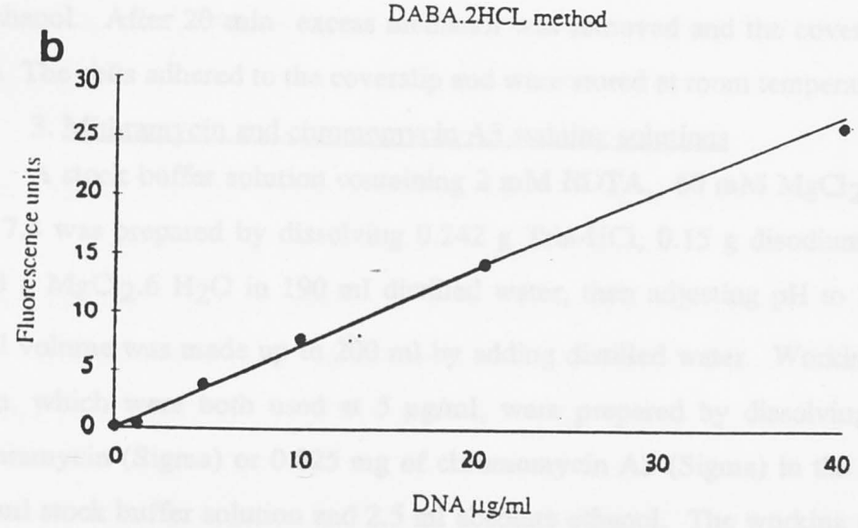
b. diaminobenzoic dihydrochloride (DABA.2HCl) reaction

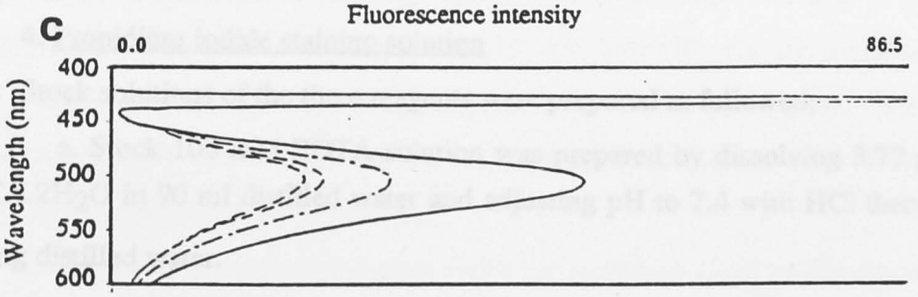
Standards were prepared as described in 6(ii).

c. profile illustrating identical excitation-emission pattern of standard salmon sperm DNA and DNA extracted from *Chlamydomonas* cells assayed by DABA.2HCl reaction.

(—) solid line represents standard; (⇢) dotted line represents DNA extracted from *Chlamydomonas*.


Diphenylamine assay
a
OD 600
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0.0
0
10
20
30
40
50
DNA µg/ml


DABA.2HCL method
b
Fluorescence units
30
25
20
15
10
5
0
0
10
20
30
40
DNA µg/ml


Fluorescence intensity
c
0.0
86.5
Wavelength (nm)
400
450
500
550
600

b. Quantitative fluorescence microscopy of nuclear DNA

(i) Preparation of materials

1. Standard chicken red blood cells (RBC)

Chicken RBC were suspended in citrate anticoagulant. The citrate was used at 0.1 M and was prepared by dissolving 3.8 g of trisodium citrate.11 H_2O or 3.1 g of trisodium citrate.2 H_2O in 99 ml distilled water and adding 1 ml of 10 % sodium azide. Chicken red blood cells were obtained from a 20 day old chick and mixed with citrate anticoagulant in the ratio of 1 volume citrate to 5 volumes blood. This mixture, if kept at 4°C, remained usable for 1 week.

2. Spreading RBC on coverslips

A small drop of RBC suspension was placed on a corner of a clean coverslip and then spread out over the whole surface in a thin layer using a new coverslip as a spreader. This thin smear of RBC was allowed to dry at room temperature and then flooded with 100 % methanol. After 20 min excess methanol was removed and the coverslip was allowed to dry. The cells adhered to the coverslip and were stored at room temperature in a sealed box.

3. Mithramycin and chromomycin A3 staining solutions

A stock buffer solution containing 2 mM EDTA, 60 mM $MgCl_2$, 10 mM Tris- HCl pH 7.4 was prepared by dissolving 0.242 g Tris-HCl, 0.15 g disodium.EDTA.2 H_2O and 2.43 g $MgCl_2$.6 H_2O in 190 ml distilled water, then adjusting pH to 7.4 with HCl. The final volume was made up to 200 ml by adding distilled water. Working solutions of each stain, which were both used at 5 µg/ml, were prepared by dissolving either 0.1 mg of mithramycin (Sigma) or 0.025 mg of chromomycin A3 (Sigma) in the solution containing 2.5 ml stock buffer solution and 2.5 ml absolute ethanol. The working solutions were kept in darkness at -20°C. Contact with both solutions was avoided because of potential hazard of mutagenesis.

4. Propidium iodide staining solution

Stock solutions of the three reagents were prepared as followed;

a. Stock 100 mM EDTA solution was prepared by dissolving 3.72 g of disodium EDTA.2H_2O in 90 ml distilled water and adjusting pH to 7.4 with HCl then to 100 ml by adding distilled water.

b. Stock 500 mM Tris-HCl pH 7.4 was prepared by dissolving 12.1 g Tris-HCl in 180 ml distilled water and adjusting pH to 7.4 with HCl. The final volume was made up to 200 ml by adding distilled water.

c. Stock 500 µg/ml propidium iodide in 50 % ethanol was prepared by dissolving 0.0025 g of propidium iodide (Sigma) in 5 ml of 50 % ethanol and was stored in darkness at -20°C. The solution was handled using rubber gloves because it is a potent mutagen.

Working solution of propidium iodide, final volume of 2.5 µg/ml, was made by mixing 100 µl Tris-HCl pH 7.4, 50 µl of 100 mM EDTA with 2.4 ml distilled water and adding 2.5 ml of absolute ethanol, then 25 µl of the stock 500 µg/ml propidium iodide was added to this buffer solution. The working solution was kept in darkness at -20°C.

5. RNAse solution

Stock solutions of three reagents were prepared as followed;

a. Stock 500 mM Tris-HCl pH 7.4 was prepared as described in 4.b.

b. Stock 150 mM NaCl was prepared by dissolving 0.88 g NaCl in 100 ml distilled water.

c. Stock heat treated (10x) RNAse solution was prepared by mixing 40 µl of 500 mM Tris-HCl pH 7.4, 200 µl of 150 mM NaCl, 1760 µl distilled water and 0.02 g of RNAse (bovine pancrease, type 1A crystallised) then immersing in a boiling water bath for 15 min. Aliquots of 500 µl stock solution were dispensed into eppendorf tubes and kept at -20°C.

The RNAse working solution was made by mixing 40 µl of 500 mM Tris-HCl pH 7.4, 200 µl of 150 mM NaCl, 1560 µl of distilled water and 200 µl of stock heat treated RNAse.

(ii) Staining procedures

Cells were harvested by centrifugation and fixed in ethanol for decolorisation and storage as described in Methods. 4.a. Before staining the cells were rehydrated and then pretreated with RNAse by washing with 10 mM Tris-HCl pH 7.4 resuspending in 100 µl RNAse working solution and incubating at 37°C. After 30 min the enzyme treated cells were centrifuged at 3000 rpm for 1 min, the pellet was washed with 10 mM Tris-HCl pH 7.4 and resuspended in 50 % ethanol.

Cells were mounted onto slides by spreading 10 µl of the enzyme treated cells onto cover slips carrying fixed RBC. The cover slip was allowed to dry at room temperature then flooded with fluorochrome working solution and left in a dark, humid

chamber for at least 3 h before removal of the excess dye. This coverslip was dried at room temperature then it was placed centrally on a slide using a drop of glycerol or Antifade solution (AF1; City University, London). The edge of the coverslip was ringed with nail vanish and the slide was kept in darkness at 4°C until viewed by fluorescence microscopy

(iii) Quantitative measurement of nuclear DNA fluorescence in serial planes of focus using a confocal microscope

The DNA content of individual nuclei was determined by (a) collecting a series of measurements of fluorescence at the different planes of focus, then (b) fluorescence intensity and area of the nuclear signal and of the surrounding cytoplasm in the plane of focus were measured, finally (c) the total DNA content of the three-dimensional nucleus was computed from the data obtained in each plane of focus at known spacings. These three stages are described here under headings (iv), (v) and (vi).

Observations and analysis of the fluorescence samples were made with a confocal laser scanning microscope MRC-500 (Bio-RAD). This microscope was equipped with mechanical stage which was attached to a stepping motor. The laser was an argon-ion type. The images were stored in the data station or backed up on a 3.5" microdisk (2S/HD, Verbatim). Samples were examined with an oil immersion 60x objective lens. For samples stained with propidium iodide, the high sensitivity green excitation filter set was used (GHS:Bandpass filter 514 DF10, Dichroic Reflector DR 540 LP, Emission filter OG 550 LP). For samples stained with mithramycin or chromomycin A3, the high sensitivity blue excitation filter set was used (BHS:Excitor filter 488 DF 10, Dichroic Reflector 510 LP, Emission Filter OG 515 LP).

(iv) Obtaining a series of optical sections

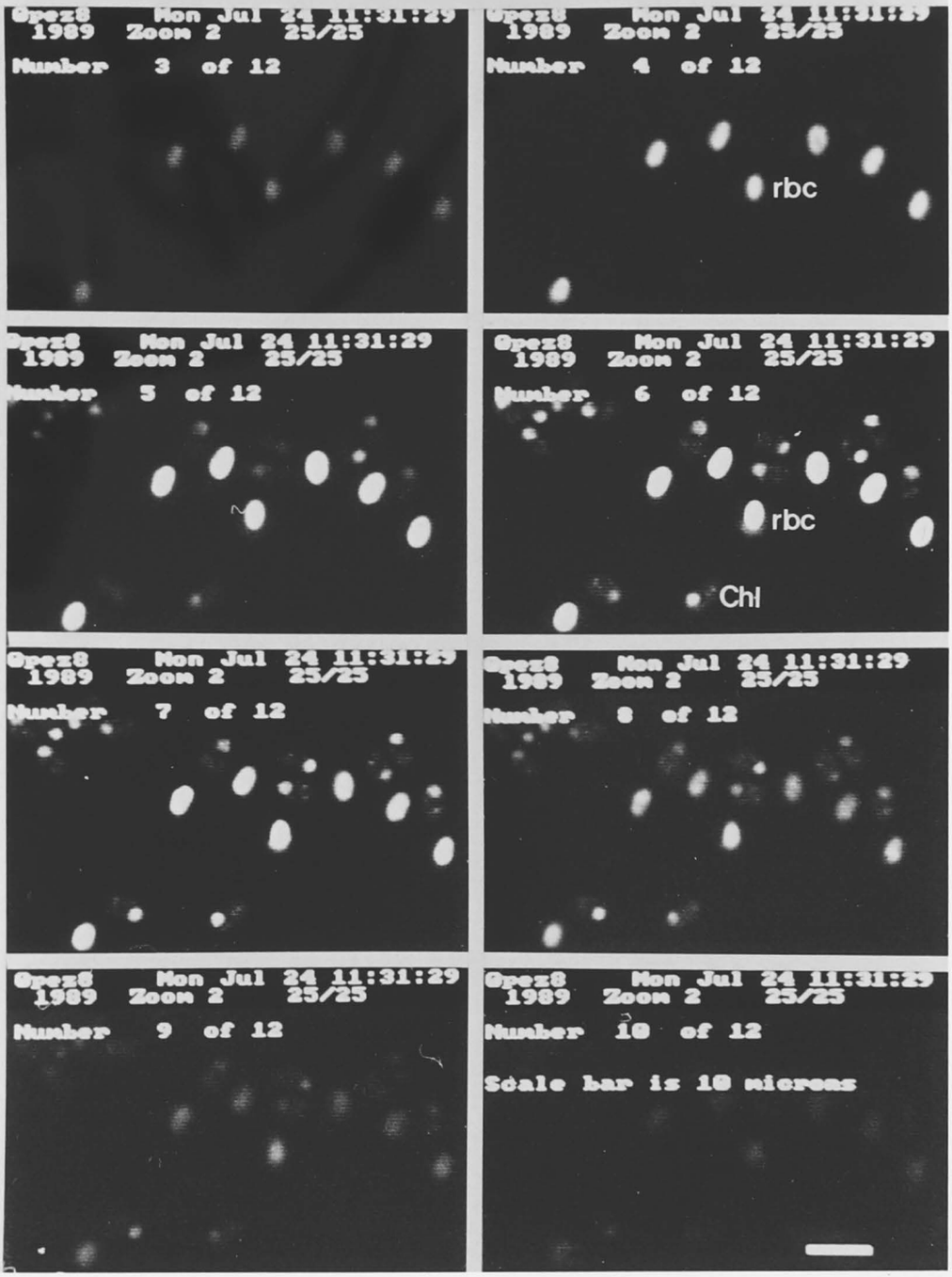
The distance between focal planes measured in a series for each sample was set at an appropriate distance between 0.7 μm to 1.5 μm depending on the size of the nucleus. It was arranged that planes of focus began above nuclear level then passed completely through and finished below each nucleus. This was usually achieved by a series of nine serial planes of focus but a larger number of planes of focus was employed if necessary, for example to accommodate the larger nuclei of the unusually large division-blocked mutant cells. Scanning factors were set as follows; zoom factor was set at either

1.5 or 2.0, corrected lens magnification was set at 60, scan speed was set at F2 level. Signals for one image were accumulated up to 25 frames to obtain the best average image and optimum signal to noise ratio. To obtain a series of optical sections, the initial focal plane was set at the level above the RBC which were distinctive because they are larger than the *Chlamydomonas* nuclei. The system was then initiated by striking the enter key. A series of images obtained by the programmed automatically stepping of the mechanical stage to the desired depth of field and from each field the sum of 25 scans was averaged. Images were displayed on the VDU screen simultaneously with each scanning. Images were stored in the data station or backed up onto a microdisk.

Fig. 2.6.2 Series of optical sections of *C. reinhardtii* using a confocal microscope MRC-500.

A series of optical images of *C. reinhardtii* cells (Chl) taken from synchronous culture at early G1 phase (0 h) mixed together with chick RBC (rbc). The optical sections were obtained by automatically stepping at the intervals of 0.7 μm. Zoom factor 2 with scanning speed F2 were used and the signals for one image were accumulated up to 25 frames to minimize noise. The initial plane of focus was above nuclear level then the series passed completely through and finished below each nucleus.

2.6


Opez8 Mon Jul 24 11:31:29 1989 Zoom 2 25/25
Number 3 of 12
Number 4 of 12
rbc
Number 5 of 12
Number 6 of 12
rbc
Chl
Number 7 of 12
Number 8 of 12
Number 9 of 12
Number 10 of 12
Scale bar is 10 microns

(v) Measurement of nuclear DNA fluorescence in serial planes of focus

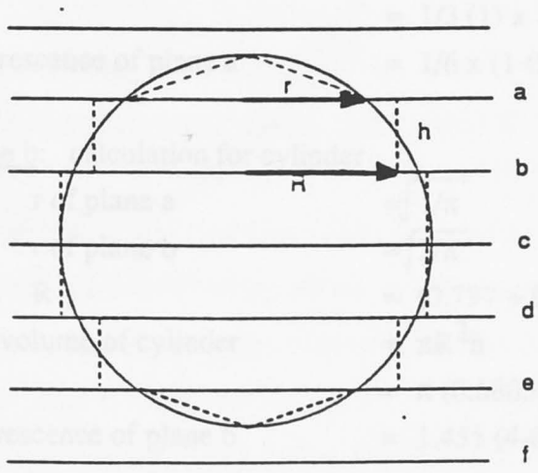
To measure fluorescence intensity of nucleus and cytoplasm, images of cells were retrieved from the data station and displayed enlarged to the full size on the computer screen. In *Chlamydomonas* cells, the nucleus is approximately spherical and is located towards the anterior of the cell whereas in RBC, the nuclei were brighter of oval shape and located centrally (Fig. 2.6.2). To measure fluorescence, a programme "area" provided by the MRC-500 software package was selected. The shape of the nucleus was drawn by carefully moving the mouse to outline the area. When the required shape was completed, mean values of the intensity and shaded area of a nucleus were displayed. To obtain background fluorescence values, the shape of the whole cell and the shape of the nucleus were outlined continuously. When this has been done, the fluorescence intensity of the whole cell excluding the nucleus was displayed. This procedure was used to exclude other potentially interfering areas, such as chloroplast or pyrenoid, by drawing around those areas. When every cell in the first plane of focus had been measured the next plane of focus was chosen. Data were recorded for individual nuclei and this area and intensity analysis was repeated until the planes of focus had progressed through all nuclei and complete set of measurements had been obtained for each.

(vi) Computation of total DNA signal in whole nuclei by three dimensional reconstruction

To calculate the total fluorescence value of nuclei, a three dimensional image of each nucleus was reconstructed. Consecutive focal planes through a nucleus corresponded to serial optical sections and the spacing between each optical sections was set as described in (iv) above. The following sequence of calculations was performed. The radius in μm of the circle was calculated from the area (μm^2) that was recorded during image analysis of the current plane of focus. The volume of each section was calculated as truncated cylinders except the top and the bottom sections which were estimated as cones (as shown in following diagram). The geometrical formulae were calculated as follows ;

volume of cone = $1/3.\pi r^2. h/2$; where r = radius of a circle = area/π, h = spacing between each focal plane

volume of tapering cylinder = $\pi R^2. h$; where R = 1/2 (r of plane a + r of plane b) and h as above



..... = calculated outline

_____ = actual outline

fluorescence value of each section = volume x net intensity ; where net intensity was obtained from an intensity of each nucleus after subtracting of the average intensity of the background in that section or without background subtracted.

Total fluorescence content of nucleus = summation of the fluorescence content from all sections passing through the nucleus.

Example; Usually the distance between planes of optical section was set so that at least six sections were taken through each nucleus, however for brevity of presentation a hypothetical situation, in which three thick optical sections transverse a nucleus, can be presented.

The spacing between each section was 1 μ. Data obtained from the program "area" was recorded as follows;

plane	nuclear area	nuclear intensity	cytoplasm intensity
a	1	1	0.5
b	2	4	0.5
c	1	1	0.5

plane a: volume of cone $= 1/3\ (\pi r^2) \times h/2$

$= 1/3\ (1) \times 1/2 = 1/6$

fluorescence of plane a $= 1/6 \times (1-0.5) = \underline{0.083}$ unit

plane b: calculation for cylinder

r of plane a $= \sqrt{1/\pi} = 0.564$

r of plane b $= \sqrt{2/\pi} = 0.797$

R $= (0.797 + 0.564)/2 = 0.6805$

volume of cylinder $= \pi R^2 h$

$= \pi\ (0.6805)^2(1) = 1.455$

fluorescence of plane b $= 1.455\ (4-0.5) = \underline{5.0925}$ unit

plane c: volume of cone $= 1/3\ (\pi r^2) \times h/2$

$= 1/3\ (1) \times 1/2 = 1/6$

fluorescence of plane c $= 1/6 \times (1-0.5) = \underline{0.083}$ unit

total fluorescence $= 0.083 + 5.0925 + 0.083 = \underline{5.2585}$ unit

Repetition of this calculation was facilitated by a computer program developed by Nicholas P. John

The relative DNA level was evaluated as a percentage of the fluorescence of the adjacent standard RBCs on the same cover slip.

RBC were used as an internal test, of the consistency of staining and of measurement in individual preparations, rather than as a calibration standard to estimate the absolute weight of DNA in the *Chlamydomonas* nucleus. The measurements in this thesis were designed to determine whether the DNA content of arrested mutant cells corresponded with that of cells known to be in G1 or in G2 phase. However if it is assumed that stained DNA of *Chlamydomonas* and chick fluoresce with equal efficiency, the relative intensity can be used to calculate the amount of *Chlamydomonas* DNA. It was found that this form

of calculation indicated amounts of DNA that are precisely in the range of DNA content that has been expected by chemical assay in *Chlamydomonas* (section IV.b).

7. Indirect immunofluorescence stains

Mutants cells were characterized in terms of their terminal arrest phenotypes by microscopic inspection of cytoskeleton development and levels of phosphorylated nonhistone proteins. These were studied by indirect immunofluorescent light microscopy.

a. Preparation of reagents

1. Stock 10x phosphate buffered saline (PBS) pH 7.4 was prepared by dissolving 80 g NaCl, 2 g KH_2PO_4, 2 g KCl, 11.5 g $Na_2HPO_4.2H_2O$, 11.4 g EGTA and 20 µl of 10 % sodium azide in 800 ml distilled water and adjusted pH to 7.4 by adding 10 M NaOH. Final volume was adjusted to 1000 ml by adding distilled water. This solution was kept at 4°C

2. 1x PBS pH 7.4 was prepared by mixing 100 ml of 10x phosphate buffer to 900 ml distilled water. This solution was kept at 4°C.

3. Microtubule stabilizing buffer (MTSB) pH 7.4; 50 mM PIPES, 5mM EGTA, 1 mM $MgSO_4$, was prepared by dissolving 15 g PIPES, 1.9 g EGTA and 0.25 g $MgSO_4.7H_2O$ in 800 ml distilled water and adjusted pH to 7.4 by adding 10 M NaOH. The final volume was made up to 1000 ml by adding distilled water. This solution was kept at 4°C and discarded every two weeks.

4. Fixing solution of 8% paraformaldehyde was prepared by dissolving 0.8 g paraformaldehyde (Sigma) in 9 ml MTSB. The solution was dissolved using medium heat. After the solution was cooled to about 30°C, 1 ml of dimethylsulfoxide (DMSO) was added. This solution was prepared freshly before use.

5. Extraction solution of 3% nonidet (NP-40) and 5% DMSO was prepared by mixing 0.3 ml NP-40 and 0.5 ml DMSO in 9.2 ml MTSB. This solution was prepared freshly before use.

6. Fresh autolysin solution was prepared by culturing wild-type cells of both mating types (mt^+ and mt^-) separately in -NHSM medium for 18-24 h. After that cells from both cultures were centrifuged at 5000 rpm and resuspended in the -NHSM medium to the density of 1-5 x 10^7 cells/ml. Then both cell suspensions were mixed together and left at 21°C under continuous light without agitation. After 30 min, cells were centrifuged at 8000

rpm at 4°C for 10 min. Cell pellet was discarded and the supernatant, which contained autolysin was kept at 4°C. The autolysin solution was prepared freshly before use.

7. Poly-L-lysine solution (1 mg/ml) was prepared by dissolving 5 mg poly-L-lysine (Sigma; P-1524, hydrobromide) in final volume of 5 ml distilled water and adjusting pH to 8.5 by adding 0.1 M KOH. Aliquots of 1 ml were kept at -20°C.

8. Stock 0.2 mg/ml DAPI solution was prepared by dissolving 0.2 mg 4,6-diamidino-2-phenylindole (DAPI) dihydrochloride (Sigma) in 1 ml 1x PBS. This solution was kept in darkness at -20°C. A working solution was prepared at 0.2 µg/ml in PBS pH 7.4.

9. Antibodies for immunolabelling

Anti-centrin polyclonal antibody was kindly provided by Prof. J.L.Salisbury, Dept. of Biochem. and Mol.Biol., Mayo Clinic, Rochester, Minnesota, U.S.A.

Anti MPM-2 and MPM-13 monoclonal antibody was kindly provided by Prof. P.N.Rao Dept. of Medical oncology, Univ. of Texas, Houston, Texas, U.S.A.

Anti-ß-tubulin monoclonal antibody was purchased from Amersham Australia, Pty.Ltd.

Anti-acetylated tubulin antibody was kindly provided by G. Piperno, The Rockefeller University, N.Y., U.S.A.

FITC-sheep antimouse (SAM) and FITC-sheep antirabbit (SAR) were purchased from Amersham.

10. Precoated slides were prepared by placing a small drop of poly-L- lysine solution (7, above) on a multiwell slide (Carlson Scientific INC.ILL, U.S.A.) and spreading thinly and evenly using a paint brush.

b. Cell growth and immunofluorescence staining

Cells were grown in TAPYPP medium at 21°C with continuous illumination and shaking. In mid-exponential phase, a portion of the culture was shifted to 33°C. After 24 h cells were harvested by centrifugation at 5000 rpm for 5 min and processed through the procedures as described below;

Anti-ß-tubulin and anti-centrin labelling; cells were suspended in fixing solution (8% paraformaldehyde in MTSB, 4. above) for 30 min then washed three times with MTSB. Pellets were suspended in fresh autolysin solution and left at 25°C with continuous shaking. After 1 h cells were washed twice with MTSB and suspended in the extraction solution (5.

above). Cells were extracted for 3 h at 25°C then washed three times with MTSB and resuspended in 20-30 µl of MTSB. A small volume of cell suspension (5 µl) was spreaded on a precoated multiwell slide (10, above). The slide carrying fixed cells was allowed to dry briefly at room temperature then immersed in -20°C methanol for 10 min for permeabilization. Before reacting with primary antibodies, cells were immersed in PBS for 5 min to rehydrate. The slide was allowed to dry briefly and 10 µl of diluted primary antibody (either anti-ß-tubulin or anti-centrin antibodies) was placed in the wells. The slide was left overnight at 25°C in a moist chamber. The anti-centrin antibody was used at 1:50 dilution in PBS + 3% BSA and anti-ß-tubulin antibody was used at 1:200 antibody dilution in PBS + 3% BSA.

After incubation with primary antibodies, the slide was washed three times with PBS pH 7.4, dried briefly then incubated with secondary antibody. For anti-ß-tubulin, FITC-SAM was used at 1:40 dilution. For anti-centrin, FITC-SAR was used at 1:40 dilution. The preparation was incubated with secondary antibody for 1 h at 37°C in a moist chamber, then washed twice with PBS pH 7.4 and immersed in a DAPI solution (0.2 µg/ml) for 10-15 min. Cells were washed once with PBS pH 7.4, dried, mounted with antifading solution (AF1; City University, London) covered with coverslip and sealed with nail vanish.

MPM-2 and anti-acetylated tubulin labelling; cells were treated as described above but omitting the use of extraction solution. Primary antibody for acetylated tubulin was used neat. MPM-2 antibody was used at 1:800 dilution in PBS + 3% BSA. The secondary antibody used for both preparations was FITC-SAM (1:40 dilution).

MPM-13 labelling; cells were washed with PBS once and resuspended in 20-30 µl PBS. A small amount (10 µl) of the cell suspension was spread onto a precoated multiwell slide, air-dried and immersed in -20°C methanol for 10 min. The slide was dried briefly then covered with fresh autolysin and incubated at 25°C for 1 h. Cells were washed gently with PBS pH 7.4, dried briefly and incubated with primary antibody (MPM-13 antibody, dilution 1:800 in PBS + 3% BSA) overnight in a moist chamber at 25°C. Then cells were processed by washing with PBS prior to application of the secondary antibody (FITC-SAM; 1:40 dilution) and DAPI staining as described above.

8. Genetic Analysis

a. Gametogenesis, mating, zygospore maturation

Induction of gametogenesis in *C. reinhardtii* was obtained by withdrawal of nitrogen from the medium (Sagar and Granick,1954; reviewed by Harris, 1988). Progression in the sexual cycle of this organism occurs in a well ordered sequence. Gametes of opposite mating types (mt^+ and mt^-) first interact via sex type-specific glycoproteins termed agglutinins, which are associated with the flagella surface. After agglutination, the sequence of the following events ensues; flagellar tip activation, cell wall loss, mating structure activation and cell fusion. The result is a quadriflagellated binucleate cell which ultimately resorbs its flagella and differentiates as a non-motile, thick-walled diploid zygote termed a zygospore (reviewed by Pasquale and Goodenough, 1987).

The zygospore matures by enlarging, darkening and developing a thick resistant wall. The resulting zygospore remain dormant in this condition for years. The zygospore can be germinated by transfer to complete medium, which contains nitrogen, and will develop by meiotic division within 24 to 36 h resulting in four to eight offspring.

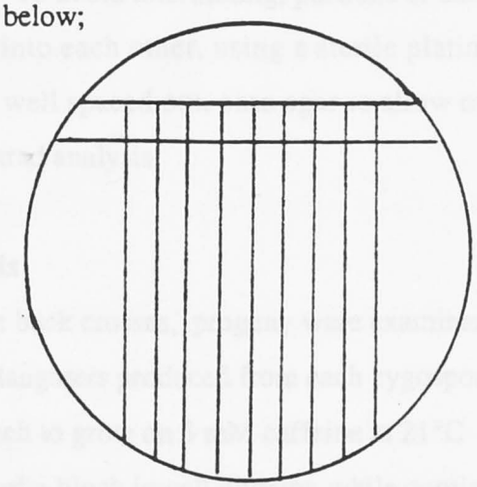
Wild-type of cells mating type plus (mt^+) and of mating type minus (mt^-) as well as mutants of unknown mating type were grown in TAPYPP medium to exponential phase. For induction of gametogenesis cells were washed twice with -NHSM medium. Pellets were resuspended in -NHSM medium to the density of approximately 1 x 10^6 cells/ml and incubated at 21°C with continuous illumination and shaking. After 18 to 24 h, an equal number of mt^+ and mt^- cells were mixed together. Each mutant was mixed with wild-type mt^+ and with mt^-. The mixtures were left in the light at 21°C without agitation. Portions of 0.2 ml were plated out onto -NHSM plates containing 4% (w/v) agar after 1.5, 3, 6 and 24 h. It was found convenient to plate successive samples onto the same agar plate. Plates were incubated for few hours or overnight until the surface had become dry. Then they were inverted and incubated under light (200 $\mu Em^{-2}s^{-1}$ PAR) at 21°C for 2 to 3 days to begin maturation before transfer to dim-light. The period of good light was required to produce zygospores with high germination rates.

The maturation of zygotes was completed during 4 to 7 days in dim-light. During the maturation period, the zygotes enlarged and the zygotic wall thickened making them easily distinguishable from surrounding unmated cells. At this stage they were called

zygospores. The zygospores could be kept dormant on the nitrogen-free agar plates in dim light at 21°C until required for germination since both light and nitrogen were essential for germination (VanWinkle-Swift, 1977).

b. Germination

For germination, mature zygospores were transferred onto a nitrogen-containing plates (TAPYPP plates). For convenience, the germination plate was marked into 8 lanes of 0.5 cm apart as shown below;



Before transfer of zygospores, the surrounding unmated cells were scraped away gently using a sterile razor blade. Zygospores tended to stick to the 4% (w/v) agar which facilitated discarding vegetative cells. Individual zygospores were located using a light microscope which was set up in a laminar flow hood. A small drop of -NHSM medium was then placed on them. Using a fine open end microcapillary tube, which had been pulled by a microelectrode puller and sterilised by dipping into chloroform and air-drying, each zygospore was sucked up by capillary motion. Several zygospores from a particular cross were then blown out onto a small square drawn at the top of each lane of the germination plate. After the liquid had dried, the germination plate was inverted over chloroform for 30 sec to kill any unmated vegetative cells. One to three zygospores were moved to the top of each lane using a closed-ended capillary as a moving rod, which was sterilised by dipping into chloroform, air-dried and held in a micromanipulator. The zygospores on TAPYPP plates were exposed to light at 21°C. After 18 h each zygospore was checked under the microscope for the release of progeny. The checking was repeating at 2 h intervals to make sure that the early stage of release was detected. Daughters about to be released could sometimes be released by gentle contact but no force was employed. If release did not occur during the period of observation, the zygospores

were placed at 4°C overnight and observation continued the following day. When daughter cells ruptured the zygospore sac they were separated using a closed-ended capillary held in the micromanipulator as a moving rod. Each daughter was separated by at least the width of one microscope field from other cells to avoid overlap of the microcolonies that grew from each of the cells. The plate was incubated at 21°C with continuous illumination until each daughter formed a visible microcolony, which usually took about 7 to 14 days. To avoid intermixing, portions of each microcolony were taken before they had grown into each other, using a sterile platinum loop. The individual progeny were streaked, well spaced out, onto agar to allow more extensive growth and subsequent testing for tetrad analysis.

c. Tetrad analysis

For scoring of the back crosses, progeny were examined for the following;

a. number of daughters produced from each zygospore

b. ability of each to grow on 3 mM caffeine at 21°C

c. occurrence of a block in cell division while continuing growth in cell size at 33°C on TAPYPP agar plates

d. cell number increase in liquid medium with and without supplementation by cAMP or db-cAMP and control supplements

Each of the progeny was examined for temperature-sensitivity by streaking very lightly onto TAPYPP plate and incubating at 33°C. Caffeine resistance was tested by streaking on 3 mM caffeine plates and incubated at 21°C. After 24 to 48 h, plates incubated at 33°C were examined for colonies with non-dividing large cells indicative of division block. After 7 to 10 days, the corresponding colonies were examined for the ability to grow on the caffeine containing agar plates. The progeny that were temperature sensitive on agar plates were further tested in liquid medium. Each was grown in TAPYPP medium at 21°C until exponential phase. The cell suspension was then distributed into a set of 30 ml universal bottles, which contained the following supplements, 5mM cAMP, 1 mM cAMP, 5 mM AMP, 1 mM AMP, 0.5 mM db-cAMP, 1 mM db-cAMP, 0.5 mM butyrate and 1 mM butyrate (Methods 5.e). After 72 or 120 h incubation, 1 ml aliquot of each sample was fixed with 0.20% final concentration of formaldehyde and cell numbers were counted using a haemocytometer as described in Methods 3.a.

9. Enzyme Assays

a. Phosphodiesterase assay

Phosphodiesterase was assayed by measurement of radioactive adenine released by enzyme action from radioactive cAMP. The reaction described more fully in section **(iii)** below.

(i) Preparation of reagents

1. 1 M Tris-HCl pH 7.5 was prepared by dissolving 24.2 g Tris-HCl in 180 ml distilled water and adjusting pH to 7.5 with HCl. The final volume was made up to 200 ml by adding distilled water.

2. 1 M $MgCl_2$ was prepared by dissolving 101.65 g of $MgCl_2.6H_2O$ in 500 ml distilled water.

3. 1 M $CaCl_2$ was prepared by dissolving 73.5 g of $CaCl_2.2H_2O$ in 500 ml distilled water.

4. 10 mg/ml bovine serum albumin (BSA) was prepared by dissolving 100 mg BSA in 10 ml distilled water. The solution was kept frozen at -20°C.

5. 100 µg/ml sheep brain calmodulin was prepared by dissolving 100 µg sheep brain calmodulin (kindly provided by Dr. P. Jablonski) in 1 ml 50 mM Tris pH 7.5.

6. 10 mM cAMP was prepared by dissolving 36.9 mg Adenosine 3',5'- cyclic monophosphate (cAMP) sodium salt (Sigma) in 8 ml distilled water. pH was adjusted to 7.0 by adding Tris-HCl. The final concentration was adjusted by adding distilled water to the final volume of 10 ml.

7. 2 mg/ml snake venom nucleotidase was prepared by dissolving 2 mg of lyophyllized venom, *Ophiophagus hannah*, (Sigma) in 1 ml distilled water. The solution was kept at 4°C.

8. 0.5 N HCl was made from 42.3 ml of 11.81 M concentrated acid in 500 ml distilled water.

9. 0.5 N NaOH was prepared by dissolving 10 g of NaOH pellet in the final volume of 500 ml distilled water.

10. Anion-exchange resin AG-1-X2, 200-400 mesh (chloride form; Bio-Rad was washed with 0.5 N HCl, 0.5 N NaOH, 0.5 N HCl and then repeatedly with distilled water

to pH 5.0. Before use, the resin was allowed to settle, the fluid was discarded then 1 part of resin was mixed to 3 parts of 100 % methanol.

11. Glass beads (type V, 400-500 microns; Sigma) were prewashed with 25 mM tris-HCl before use.

12. 100 mM phenylmethane-sulphonyl fluoride (PMSF) was prepared by dissolving 1.79 g of PMSF in 10 ml absolute ethanol, stored at 4°C and discarded after 1 week.

13. 100 mM dithiothreitol (DTT) was prepared by dissolving 84.2 mg DTT in 10 ml distilled water and stored at -20°C.

(ii) Cell growth and extraction for phosphodiesterase assay

Cells were grown at 21°C in TAPYPP medium until the culture reached exponential phase, then a portion of the culture was transferred to 33°C. After 24 h, cells were harvested by centrifugation at 5000 rpm for 10 min. Pellets were washed twice with cold 25 mM Tris-HCl pH 7.5 and resuspended in cold solution containing 25 mM Tris-HCl pH 7.5 and 1mM PMSF. Cells were homogenized by vortexing them with prewashed glass beads. Samples were mixed vigorously at full vortex speed for 3 cycles of 1 min shaking and 1 min sitting in ice. The resulting homogenates were centrifuged at low speed (1000 rpm) for 5 min to spin down the unbroken cells. Supernatants were used immediately as crude extracts for the phosphodiesterase assays.

(iii) Assay procedures

Principle of the reaction

Cyclic nucleotide phosphodiesterase was assayed using a modification of the method developed by Thompson et. al. (1979). The enzyme activity was assayed by monitoring the conversion of the radio-labelled substrate, [8-^{3}H]cAMP to the noncyclic monophosphate and then, in a later reaction catalysed by nucleotidase to adenosine. The two steps in the conversion are;

[8-^{3}H] cyclic 3',5'-AMP---^{I}-->[8-^{3}H]-AMP---^{II}-->[^{3}H]adenosine

Step I, [8-^{3}H] cAMP is hydrolysed to [8-^{3}H] 5'-AMP by cyclic nucleotide phosphodiesterase in the presence of divalent cation, Mg^{2+}or Mn^{2+}.

Step II, all the [8-^{3}H] 5'-AMP formed is converted to [8-^{3}H] adenosine by snake venom nucleotidase. The radioactive adenosine was then separated from charged nucleotides by precipitation with anion exchange resin, and leaves [8-^{3}H] adenosine as the only radioactive compound to be counted. As an excess of nucleotidase is employed, the rate-limiting step in the sequence is the activity of phosphodiesterase. Thus the amount of phosphodiesterase activity is proportional to the amount of [8-^{3}H] adenosine counted.

Assay mixture and reaction

The assay mixture for phosphodiesterase contained the following reagents in a final volume of 100 µl;

40 mM Tris-HCl pH 7.5

2 mM dithiothreitol

5 mM $MgCl_2$

60 µg BSA

1 mM $CaCl_2$

1 µg sheep brain calmodulin

20 µM cAMP

30,000 cpm [8-^{3}H] cAMP (specific activity 41.7 Ci/mmol)

cell extract

Phosphodiesterase reaction was initiated by adding a volume of cell extract containing 20-40 µg protein to the assay mixture. Each reaction was run in duplicate. Extract from cells at 21°C was tested for activity at 25°C and 35°C while extract from cells at 33°C was tested at 35°C. The enzyme was held on ice before assay. Tubes were prewarmed to assay temperature before adding enzyme then the reaction was allowed to occur for 15 min and was terminated by dipping each tube in a boiling water bath for 2 min. After the sample was cooled to 25°C, 20 µl of the nucleotidase snake venom solution (stock concentration of 2 mg/ml) was added to the reaction mixture and further incubated for another 10 min at 30°C. One ml of a slurry of resin and methanol mixture (1:3 v/v) was added and mixed well. The mixture was left on ice for 5 min and mixed occasionally. The suspension was then centrifuged at 3000 rpm for 10 minutes at 4°C. Then a volume of 0.8 ml of supernatant was taken out and mixed with 6 ml of the scintillation fluid (Emulsifier-safe; Packard instrument Company INC. ILL, U.S.A.). Samples were then counted for ^{3}H in the scintillation counter (Beckman LS 3801,

U.S.A.). For the control sample, cell extract was added to the reaction mixture, boiled immediately and treated with the same procedure as the other samples.

cAMP hydrolysed by phosphodiesterase was calculated from the equimolar amount of adenosine produced

The activity of phosphodiesterase was linear with the amount of cell extract assayed in the range of 10 to 40 μg (Fig. 2.9.1a) and linear with time over the period 5 to 25 min (Fig. 2.9.1b).

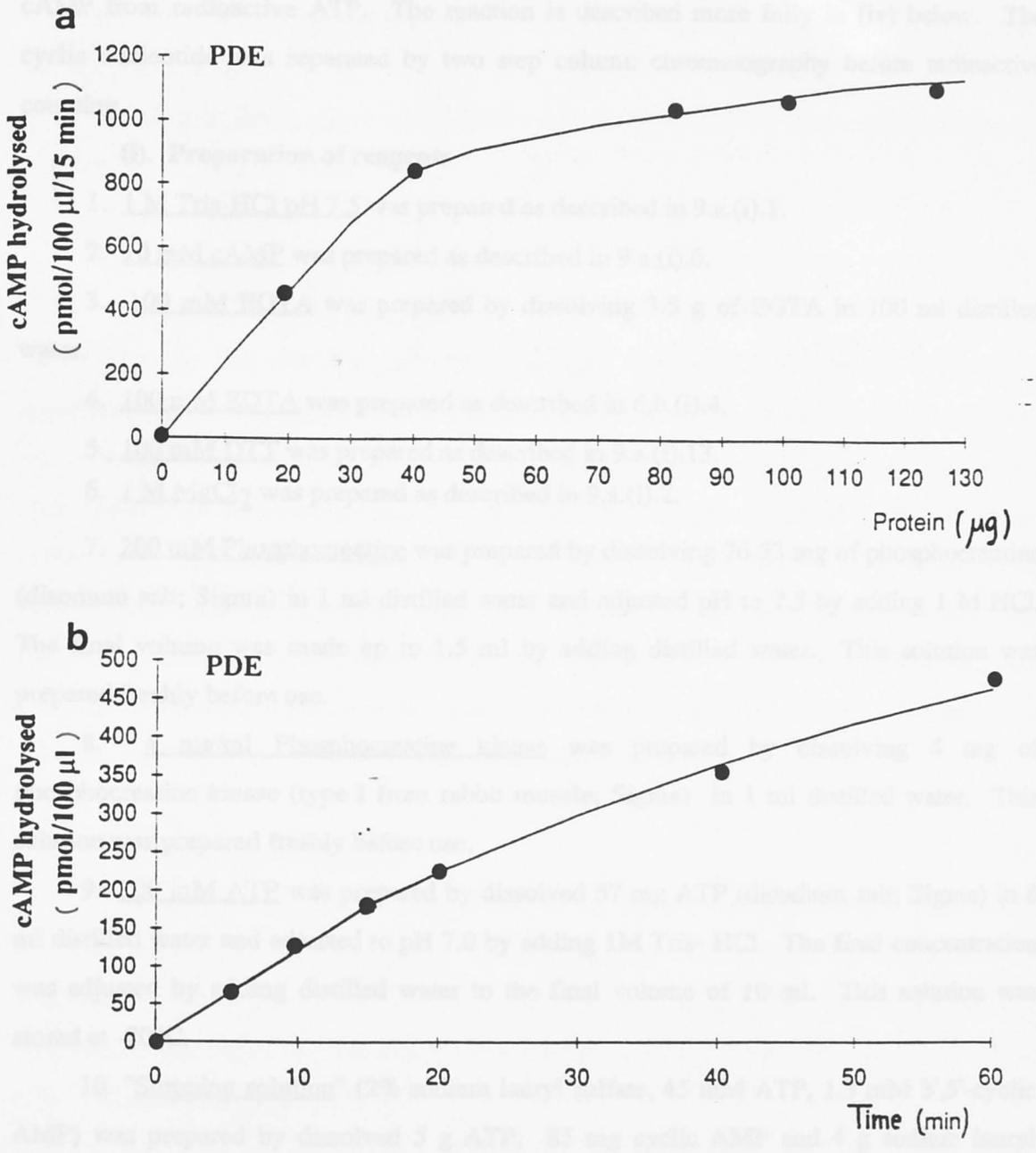
Fig. 2.9.1 Kinetics of phosphodiesterase assay

Preparation of cell extract and the reaction was performed as described in Methods 9.a.(ii) and (ii)

a. Activities of phosphodiesterase measured during 15 min of reaction due to different amounts of protein (μg) in extracts from wild-type cells.

b. Activities of phosphodiesterase over different incubation times (min) in assays containing 20 μg protein from crude cell extract.

2.9.1


a
PDE
cAMP hydrolysed
(pmol/100 µl/15 min)
1200
1000
800
600
400
200
0
0 10 20 30 40 50 60 70 80 90 100 110 120 130
Protein (µg)
b
PDE
cAMP hydrolysed
(pmol/100 µl)
500
450
400
350
300
250
200
150
100
50
0
0 10 20 30 40 50 60
Time (min)

b. Adenylate cyclase assay

Adenylate cyclase activity was estimated by measuring the formation of radioactive cAMP from radioactive ATP. The reaction is described more fully in (iv) below. The cyclic nucleotide was separated by two step column chromatography before radioactive counting.

(i). Preparation of reagents

1. 1 M Tris-HCl pH 7.5 was prepared as described in 9.a.(i).1.
2. 10 mM cAMP was prepared as described in 9.a.(i).6.
3. 100 mM EGTA was prepared by dissolving 3.8 g of EGTA in 100 ml distilled water.
4. 100 mM EDTA was prepared as described in 6.b.(i).4.
5. 100 mM DTT was prepared as described in 9.a.(i).13.
6. 1 M $MgCl_2$ was prepared as described in 9.a.(i).2.
7. 200 mM Phosphocreatine was prepared by dissolving 76.53 mg of phosphocreatine (disodium salt; Sigma) in 1 ml distilled water and adjusted pH to 7.5 by adding 1 M HCl. The final volume was made up to 1.5 ml by adding distilled water. This solution was prepared freshly before use.
8. 4 mg/ml Phosphocreatine kinase was prepared by dissolving 4 mg of phosphocreatine kinase (type I from rabbit muscle; Sigma) in 1 ml distilled water. This solution was prepared freshly before use.
9. 100 mM ATP was prepared by dissolved 57 mg ATP (disodium salt; Sigma) in 8 ml distilled water and adjusted to pH 7.0 by adding 1M Tris- HCl. The final concentration was adjusted by adding distilled water to the final volume of 10 ml. This solution was stored at -20°C.
10. "Stopping solution" (2% sodium lauryl sulfate, 45 mM ATP, 1.3 mM 3',5'-cyclic AMP) was prepared by dissolved 5 g ATP, 85 mg cyclic AMP and 4 g sodium lauryl sulfate (SDS) in 150 ml distilled water. The suspension was adjusted to pH 7.5 by adding Tris-HCl buffer. The total volume was adjusted to 200 ml by adding distilled water.
11. 1 M imidazole buffer pH 7.3 was prepared by dissolving 68 g imidazole (Sigma) in 600 ml distilled water and adjusted pH to 7.3 by adding concentrated HCl. The final volume was made up to 1 l with distilled water.

12. 0.1 M imidazole pH 7.3 was prepared by diluted 100 ml of 1 M imidazole in 900 ml distilled water.

13. 60 mM caffeine was prepared by dissolving 116.5 mg caffeine (Sigma) in 10 ml warm distilled water.

14. 60 mM theophylline was prepared by dissolving 108 mg of theophylline (Sigma) in 10 ml warm distilled water.

(ii) Preparation of columns

Packing Dowex-50 columns

Dowex AG 50W-X4 (200-400 mesh) resin (hydrogen form; Sigma) was washed repeatedly with deionized water until the effluent was colourless. Then the slurry (50% v/v) was transferred into glass wool-stoppered columns (31.5 x 0.6 cm), which were held in a rack constructed to accommodate 24 columns. The resin was added until the packed volume was approximately 5 ml then it was washed with deionized water to allow all the beads to settle and form the column bed. This column was washed with 5 ml 1 N HCl and allowed to drain. The acid-washed column was kept at room temperature. Before use, each columns was washed with deionized water (20-30 ml) and allowed to drain. For recycling columns were washed with 20-40 ml deionized water and then 5 ml 1 N HCl.

Packing alumina columns

0.6 g of dry neutral alumina (type WN-3, Sigma) was added into a glass wool-stoppered column (14.5 x 0.6 cm). The column was washed with 12-15 ml of 1 M imidazole HCl buffer, pH 7.3 followed by 15-20 ml of 0.1 M imidazole HCl buffer, pH 7.3. The column was stored at room temperature. Before use the column was washed with 8 ml of 0.1 M imidazole buffer pH 7.3.

Determination of elution volumes for ATP and cAMP

1. Dowex 50 columns

A solution containing about $1x10^4$ cpm [α-^{32}P]ATP (18 pmol) and $1x10^4$ cpm [^{3}H]cAMP (13 pmol) was made in 1 ml sample buffer (iv). This solution was decanted into a water-washed Dowex 50 column and effluent was allowed to drain completely into a vial. Then 20 ml distilled water was gradually added to the column and 1.0 ml

fractions were collected. Each vial was mixed with 6 ml scintillation fluid and the two isotopes were counted. The elution pattern of [^{32}P]ATP and [^{3}H]cAMP is shown in Fig. 2.9.2.a. [^{32}P]ATP was eluted at 6 ml while [^{3}H]cAMP was clearly resolved into later fractions. The Dowex-50 column could therefore be used to separate the substrate [^{32}P]ATP from the product [^{32}P]cAMP after the adenylate cyclase reaction.

2. Alumina columns

To determine the elution pattern of cAMP from the alumina column a sample of the cyclic nucleotide (1 x 10^4 cpm [α-^{32}P] ATP and 1 x 10^4 cpm [^{3}H]cAMP in 1 ml sample buffer) was loaded in a Dowex-50 column. The ATP was discarded in the first 6 ml that was eluted with water and cAMP was retained in the next 14 ml of eluate, as shown in Fig. 2.9.2a. The cAMP was then loaded onto the alumina column and the flow-through liquid retained in a vial. Then [^{3}H]cAMP was eluted with 10 ml of 0.1 M imidazole buffer pH 7.3 collected as twenty 0.5 ml fractions in separate vials. Each vial was mixed with 6 ml scintillation fluid and counted in the scintillation counter. The elution pattern of [^{3}H]cAMP is shown in Fig. 2.9.2b. [^{3}H]cAMP was eluted from alumina column in a 5 ml volume.

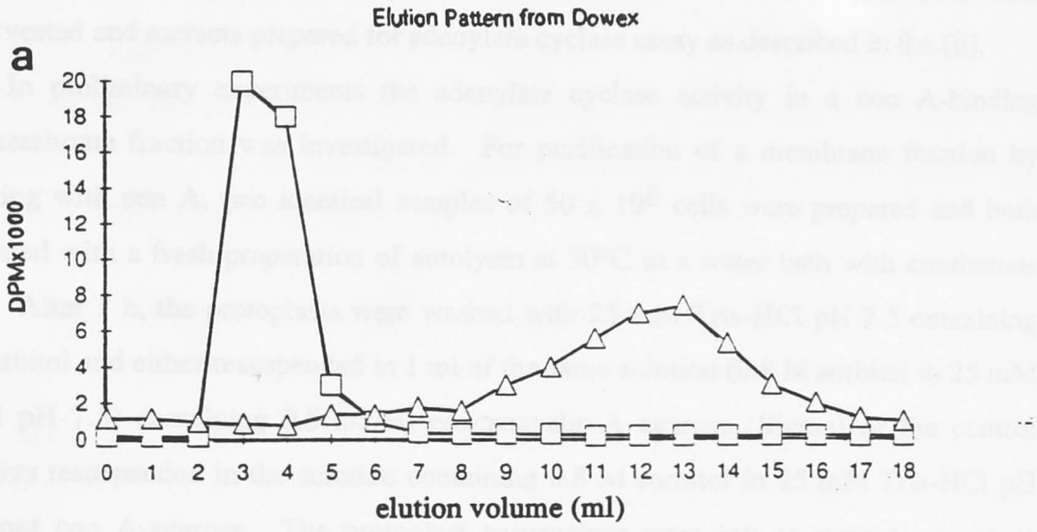
Fig. 2.9.2 Determination of elution patterns of ATP and cAMP from Dowex-50 and neutral alumina columns.

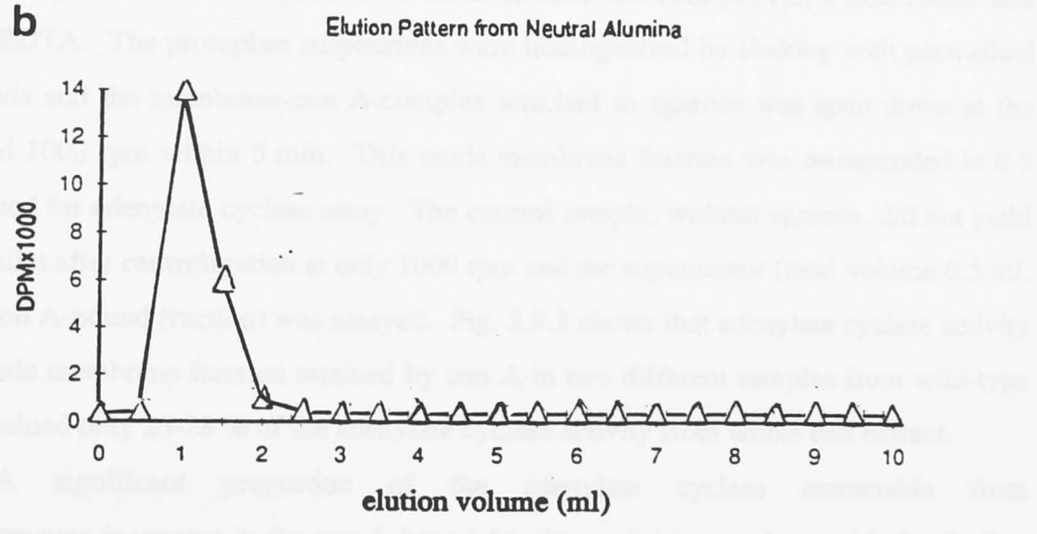
A mixture of $1x10^4$ cpm [^{32}P] ATP and $1x10^4$ cpm [^{3}H]cAMP was loaded onto a Dowex-50 column. The column was eluted with 20 ml distilled water. Pooled fractions containing [^{3}H]cAMP were further purified by loading onto neutral alumina column and [^{3}H]cAMP was eluted with 0.1 M imidazole buffer pH 7.3.

a. Elution of [^{32}P]ATP and [^{3}H]cAMP from Dowex-50 column.
(▭) represents [^{32}P]ATP.
(△) represents [^{3}H]cAMP.

b. Elution of [^{3}H]cAMP from neutral alumina column.

2.9.2


Elution Pattern from Dowex
a
DPMx1000
20
18
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
elution volume (ml)


b
Elution Pattern from Neutral Alumina
DPMx1000
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10
elution volume (ml)

(iii) Cell growth and extraction for adenylate cyclase assay

Cells were grown at 21°C in TAPYPP medium until the culture reached exponential phase, then a portion of the culture was transferred to 33°C. After 24 h, cells were harvested and extracts prepared for adenylate cyclase assay as described in 9.a.(ii).

In preliminary experiments the adenylate cyclase activity in a con A-binding plasmamembrane fraction was investigated. For purification of a membrane fraction by complexing with con A, two identical samples of 50 x 10^6 cells were prepared and both were treated with a fresh preparation of autolysin at 30°C in a water bath with continuous shaking. After 1 h, the protoplasts were washed with 25 mM Tris-HCl pH 7.5 containing 0.8 M sorbitol and either resuspended in 1 ml of the same solution (0.8 M sorbitol in 25 mM Tris-HCl pH 7.5) containing 0.5 mg/ml concanavalin-A agarose (Sigma) or the control sample was resuspended in the solution containing 0.8 M sorbitol in 25 mM Tris-HCl pH 7.5 without con A-agarose. The protoplast suspensions were left at room temperature (25°C) for 10 min to allow con A to bind to the membrane, then centrifuged at 1000 rpm for 5 min. Both pellets were resuspended 0.5 ml of 25 mM Tris-HCl pH 7.5, 1 mM PMSF and 0.1 mM EDTA. The protoplast suspensions were homogenized by shaking with prewashed glass beads and the membrane-con A-complex attached to agarose was spun down at the low speed 1000 rpm within 5 min. This crude membrane fraction was resuspended in 0.5 ml and used for adenylate cyclase assay. The control sample, without agarose, did not yield a large pellet after centrifugation at only 1000 rpm and the supernatant (total volume 0.5 ml, like the con A-bound fraction) was assayed. Fig. 2.9.3 shows that adenylate cyclase activity of the crude membrane fraction retained by con A in two different samples from wild-type cells contained only 25-28 % of the adenylate cyclase activity from whole cell extract.

A significant proportion of the adenylate cyclase extractable from *Chlamydomonas* is present in the con A-bound fraction and this correlates with the finding of some adenylate cyclase activity in purified plasma membranes of other eukaryotes. However, about three quarters of the activity is not retained by con A. It was considered most appropriate to routinely assay the total enzyme fraction rather than membrane-bound adenylate cyclase, for two reasons; first it seemed possible that the membrane-bound fraction might be dedicated to responding to external signals, perhaps mating pheromones and therefore less relevant to cell cycle progress. Second, it was considered that the assay of total adenylate cyclase would be more likely to include all of the activities that might have

been affected by mutation and also the proportion of total activity that might have been lost would be revealed.

(iv) Assay procedure

Principle of the reaction

Adenylate cyclase activity was determined essentially according to the method of Salomon (1979). The reaction converts nucleotide triphosphate (ATP) to 3',5'-cAMP and pyrophosphate in the presence of Mg^{2+} or Mn^{2+}.

Adenosine 5'-*P-P-P-------> Adenosine 3',5'-*P +PP_i

Adenylate cyclase activity was determined by measuring the conversion of radioactive [α-^{32}P]ATP to [α-^{32}P]cAMP. The reaction products and the excess substrate were separated by sequential chromatography on Dowex-50 cation exchanger and on neutral alumina columns.

Assay mixture and reaction

The conditions of the assay that were employed did not provide free calcium, therefore calcium independent adenylate cyclase activity was measured in this study.

The 100 µl reaction mixture contained the following components at the final concentrations shown below;

50 mM Tris-HCl pH 7.5

300 µM cAMP

0.1 mg/ml BSA

0.1 mM EGTA

0.1 mM EDTA

20 mM creatine phosphate

400 µg/ml creatine kinase

1mM dithiothreitol

10 mM $MgCl_2$

5 mM caffeine

0.25 mM ATP

[α-^{32}P]ATP was presented at $1x10^6$ cpm per assay at the specific activity of 3000 Ci/mmol. 50 µg of protein extract was included in each assay.

The reactions were run in duplicate and initiated by adding sample protein to the prewarmed reaction mixture and was the reaction allowed to proceed for 10 min. Extract from cells at 21°C was tested for activity at 25°C and 35°C while extract from cells at 33°C was tested at 35°C. The enzyme was held on ice before assay. As soon as the enzyme was mixed with reagents its catalytic activity could begin, therefore any reduction of catalytic activity observed at 35°C indicates rapid loss of activity. The reaction was terminated by adding 100 µl of stopping solution. After stopping, 5,000 cpm of [^{3}H]cAMP was added to each sample and used as an internal recovery standard. Samples were heated in a boiling water bath for 2 min and 0.8 ml of distilled water was added to each. [^{32}P]cAMP was isolated by decanting each sample into a Dowex 50 column (dimension of resin bed in column was 22 x 0.6 cm). The run- through together with a 5 ml wash with distilled water was discarded. Then 15 ml water was passed through the column (dimension of resin bed in the column was 3.5 x 0.6 cm) added and the eluates were pooled into a vial. Pool fractions were passed through an alumina column and the effluent of 10x1 ml was collected. Eluates were mixed with scintillation fluid and counted on a scintillation counter. The control sample was prepared by adding 100 µl of stopping solution to the reaction mixture immediately after adding cell extract and it was then processed as the other samples.

For radioactive counting, the scintillation counter (Beckman) was adjusted for simultaneous counting of ^{3}H and ^{32}P with minimal cross over.

The amount of ATP converted to cAMP by adenylate cyclase activity was calculated from the equimolar amount of radioactive [^{32}P]cAMP produced from the radioactive [^{32}P]ATP. Net cAMP from each reaction was calculated by subtracting the basal cAMP presented in the control tube with the amount of cAMP produced in the unknown sample. Total cAMP was then converted using percentage of recovery of [^{3}H]cAMP which was added as recovery marker. The percentage of recovery was approximately 70-80%. The amount of [^{3}H]cAMP added was insignificant compared with the amount of cAMP produced from the reaction.

The rate of cAMP production was approximately linear with amount of cell extract for amounts of protein between 20 to 70 µg per assay (Fig. 2.9.4).

Fig. 2.9.3 Comparison of adenylate cyclase activities of wild-type cells recovered in total cell free extract and in crude membrane fraction retained by con A agarose. The whole cell extract and membrane fractionation were performed as described in Methods 9.b.(iii). Adenylate cyclase activity was assayed as described in Methods 9.b.(iv).

(□) represents adenylate cyclase activity in total cell free extract.

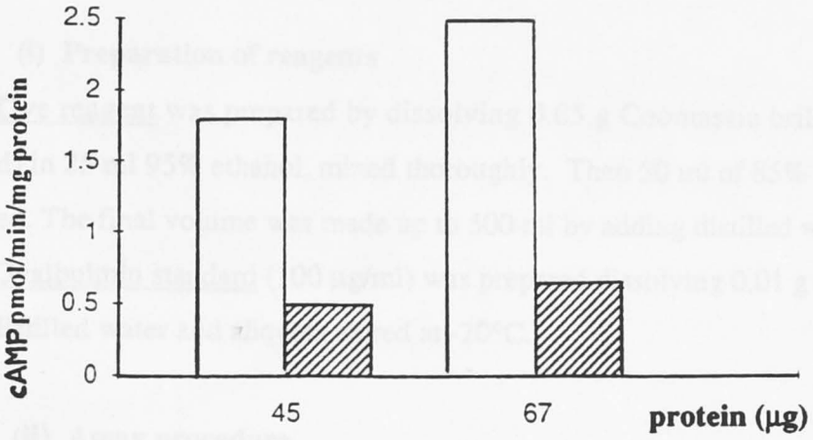
(▨) represents adenylate cyclase activity in crude membrane fraction retained by con A agarose.

x-axis represents amount of protein (μg) in the sample.

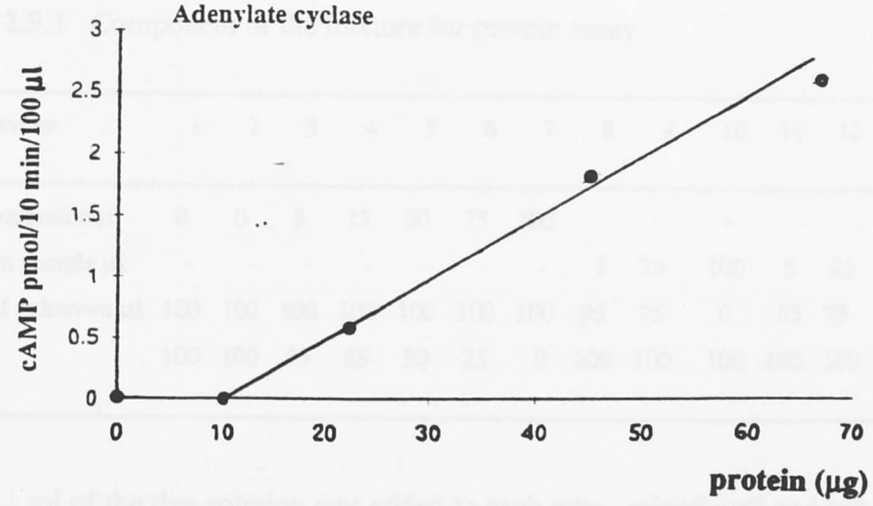
Fig. 2.9.4 Adenylate cyclase activities in crude cell free extract of wild-type cells. Cell extraction and adenylate cyclase activity were performed as described in Methods 9.b.(iii) and (iv). The reaction was assayed in the presence of different amounts of protein from crude cell extract.

2.9.3

Adenylate cyclase in crude whole cell and membrane fraction



2.9.4



c. Protein estimation

This assay method was based on the Coomassie Brilliant blue assay for protein developed by Spector (1978).

(i) Preparation of reagents

1. Dye reagent was prepared by dissolving 0.05 g Coomassie brilliant blue G250 (Bio-Rad) in 25 ml 95% ethanol, mixed thoroughly. Then 50 ml of 85% phosphoric acid was added. The final volume was made up to 500 ml by adding distilled water.

2. Ovalbulmin standard (100 µg/ml) was prepared dissolving 0.01 g of ovalbumin in 100 ml distilled water and aliquots stored at -20°C.

(ii) Assay procedure

Assays were performed in 1.5 ml eppendorf tubes. The mixture in each tube was prepared as followed;

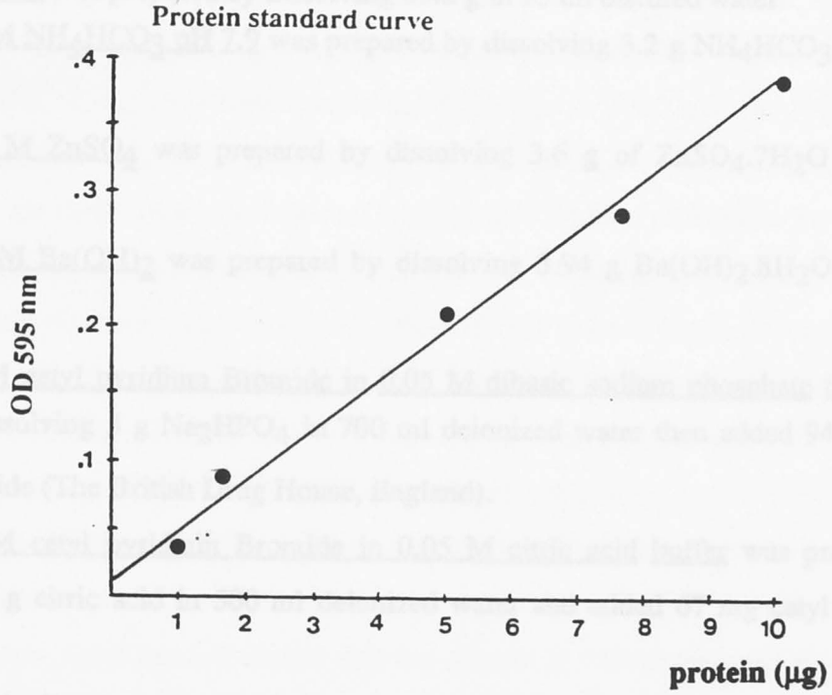
Table 2.9.1 Component of the mixture for protein assay

Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13
stock ovalbumin µl	0	0	5	15	50	75	100	-	-	-	-	-	-
unknown sample µl	-	-	-	-	-	-	-	5	25	100	5	25	100
buffer of unknown µl	100	100	100	100	100	100	100	95	75	0	95	75	0
H_2O µl	100	100	95	85	50	25	0	100	100	100	100	100	100

1 ml of the dye solution was added to each tube, mixed well and left 10-15 min at room temperature. Absorbance was read at 595 nm with a spectrofluorometer. Tubes 1 and 2 were used as blank, the contents of tube 1 being left in the spectrophotometer (reference cuvette) and zeroed against tube 2 which was poured freshly into measuring cuvette before measuring the unknowns. The protein concentration presented in each assay tube was 0, 0.5 1.5, 5, 7.5, 10 µg.

Fig. 2.9.5 Ovalbumin standard curves for protein estimation.

The preparation of the standards and unknown were described in Methods 9.c.(ii). Absorbance was read at 595 nm and the unknown was determined from the standard curve.



10. Determination of cellular cAMP by HPLC of a fluorescent derivative

cAMP was measured by formation of a fluorescent etheno-derivative by reaction with chloroacetaldehyde and separation of the derivative by HPLC using fluorescence to monitor fractionation and quantify separated components.

a. Preparation of reagents for sample preparation and HPLC fractionation

1. 6 N perchloric acid (PCA) was prepared by mixing 50.4 ml of conc PCA (71%) with distilled water to a final volume of 100 ml.

2. 1 M Tris-HCl pH 7.5 buffer was prepared as described in 9.a.(i).1.

3. 5 N KOH was prepared by dissolving 8.42 g in 25 ml distilled water.

4. 0.05 M NH_4HCO_3 pH 7.9 was prepared by dissolving 3.2 g NH_4HCO_3 in 800 ml distilled water.

5. 0.25 M $ZnSO_4$ was prepared by dissolving 3.6 g of $ZnSO_4.7H_2O$ in 50 ml distilled water.

6. 0.25 M $Ba(OH)_2$ was prepared by dissolving 3.94 g $Ba(OH)_2.8H_2O$ in 50 ml distilled water.

7. 26 µM cetyl pyridium Bromide in 0.05 M dibasic sodium phosphate buffer was prepared by dissolving 5 g Na_2HPO_4 in 700 ml deionized water then added 94 mg cetyl pyridium Bromide (The British Drug House, England).

8. 26 µM cetyl pyridium Bromide in 0.05 M citric acid buffer was prepared by dissolving 5.25 g citric acid in 500 ml deionized water and added 67 mg cetyl pyridium Bromide.

9. HPLC buffer was prepared by titrating 0.05 M dibasic sodium phosphate (7) with 0.05 M citric acid (8) to pH 4.8. The buffer was then filtered (0.45 µm filter, Milipore HA), mixed with methanol (HPLC grade) to a final concentration of 15 % (v/v), and degassed.

10. Stock 3 mM cAMP standard was prepared by dissolving 5.538 mg cAMP (sodium salt; Sigma) in 5 ml distilled water and kept frozen at -20°C. Working standards were made by diluting stock 3 mM to 0.075, 0.15, 0.3, 1.5 and 3.0 µM. These standards were kept at -20°C.

11. Neutral alumina column for preliminary purification of cAMP in cell extracts was prepared by adding 1 gm of neutral alumina (type WN-3; Sigma) into a glass wool-

stoppered column (14 x 0.6 cm). The column was washed with 20 ml deionized water and then 20 ml 0.05 M NH_4HCO_3 pH 7.9.

12. A Dowex-50 column for further purification of the alumina fraction prior to HPLC was prewashed as described in 9.b.(ii). The slurry of 50% v/v was transferred into a glass wool-stopper columns (31.5 x 1.0 cm) until the packed volume was approximately 2 ml then washed with deionized water, 1 N HCl and deionized water as described in 9.b.(ii).

b. Cell growth, cAMP extraction and partial purification by Dowex-50 and neutral alumina

Cells were grown in TAPYPP medium at 21°C with continuous shaking and illumination. At mid-exponential phase, a portion of the culture was shifted to 33°C and incubated for 24 h. Cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C and washed twice with 25 mM Tris-HCl buffer pH 7.5. Thus the cAMP in the medium was discarded. For the final wash cells were transferred to a "Falcon tube" then the pellet was suspended in a minimum volume of buffer (less than 5% of packed cell volume) and frozen dropwise in liquid nitrogen. The frozen cells were then ground in liquid nitrogen and total cell weight was estimated. For cAMP extraction, the ground cells were quickly thawed at 37°C and 6 N perchloric acid (PCA) was added to a final concentration of 0.6 N. A recovery marker comprising [8-^{3}H]cAMP (10,000 cpm) was then added. This amount of added authentic cAMP is only 10^{-15} moles and had no significant effect on the total amount of cAMP with the sample. The acid treated sample was left at 4°C for 1 h with intermittent vortex mixing then centrifuged at 10,000 rpm for 10 min at 4°C to removed the denatured cellular materials. The pH of the supernatant was adjusted to pH 7 by adding 5 N KOH. The precipitate was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was concentrated under reduced pressure at 40°C and then applied to a neutral alumina column. The flow- through liquid was discarded. The column was then eluted with 0.05 M NH_4HCO_3 pH 7.9 buffer and the first 10 ml of eluate was collected and dried under reduced pressure at 40°C. This dried sample was resuspended in 1 ml distilled water and passed through a Dowex-50 column. The flow-through and a subsequent 2 ml of distilled water wash were discarded. The column was then eluted with 10 ml of distilled water from which an aliquot of 0.5 ml was counted to determine recovery of the [8-^{3}H]cAMP. By this extraction method the overall recovery of cAMP was 15-20%.

Recoveries of less than 30% are widely reported (e.g. Frankco, 1981 and 1982). The eluates were evaporated to dryness under reduced pressure at 40°C before being redissolved in distilled water and subjected to the derivatization reaction.

As a positive control cells treated with 3 mM caffeine or 3 mM theophylline were sampled to test for an increase in internal cAMP levels.

A sample of culture was examined for microbial contamination 24 h before harvesting and at the time of harvesting by plating an aliquot of 0.2 ml on TAPYPP agar plates and incubating at 37°C overnight.

c. Derivatization of cAMP

Chloroacetaldehyde was synthesized by modification of the method of Secrist et.al., (1972); 10 ml of diluted H_2SO_4, prepared by mixing 1 ml of concentrated H_2SO_4 with 9 ml distilled water, was added to 10 ml chloroacetaldehyde dimethyl acetal (Sigma). The mixture was distilled slowly and the chloroacetaldehyde fraction was collected at 80-85°C as a liquid condensate in a water cooled condenser. This reagent was stored at -20°C.

The dried semipure cAMP sample after Dowex-50 was redissolved in 0.35 ml distilled water. A volume of 0.15 ml was taken and mixed with 0.15 ml of 0.25 M $ZnSO_4$ and 0.15 ml of 0.25 M $Ba(OH)_2$. The precipitate was removed by centrifugation at 12,000 rpm at room temperature in a microfuge for 10 min. 0.3 ml of the supernatant was transferred to a 1 ml reacti-vial (Pierce, USA) and 30 µl of chloroacetaldehyde was added. The sample tube was capped well and incubated in dry heat at 80°C for 45 min in a heating block. The reaction was stopped by placing the sample on ice then it was evaporated to dryness under reduced pressure at 40°C. Before HPLC, the dried sample was redissolved in 30-40 µl of HPLC solvent (10.a.9).

Standards of authentic cAMP were reacted with $ZnSO_4$ and $Ba(OH)_2$ in parallel with the unknown samples to determine chromatographic mobility and to establish a standard curve of fluorescence intensity.

d. HPLC

HPLC was performed on a reversed phase C-18 (10 µ) Radial-Pak cartridge (Waters, 8 x 100 mm) using equipment supplied by Waters (Milford, Mass., USA). The solvent (as described in 10.a.9) was delivered by a constant flow rate (3 ml/min) pump

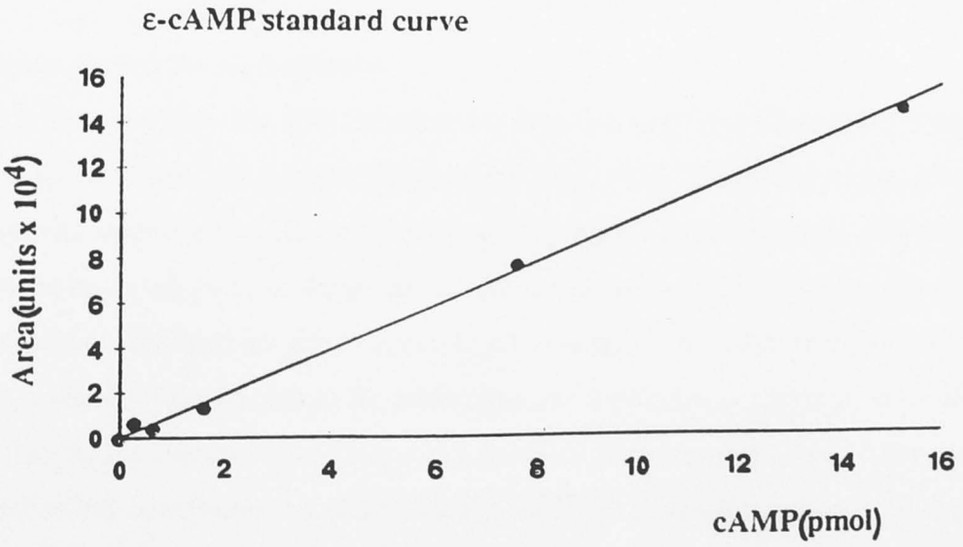
(Model M-6000A). The samples were introduced through a universal injector (Model U6K). UV absorbance was monitored at 275 nm using a programmable multiwavelength detector (Model 490). Fluorescence emission was monitored at 410 (bandwidth 15 nm) and excitation at 298 nm (bandwidth 13 nm) with a Shimadzu (Model RF-535) Fluorescence HPLC Monitor (12 µl flowcell). The system was controlled and data were acquired using Waters Maxima 820 software. Identification of individual peaks of the fluorescence chromatogram was done by comparison of the retention times of _-cAMP standards as well as by spiking the sample with standards to check which peak is increased in height and which coincides with radioactively-labelled cAMP.

Quantitation of cAMP was based on measuring peak area of the sample against standards that were processed from reaction with $ZnSO_4$ and $Ba(OH)_2$ in parallel with sample of homogenates. The calibration for cAMP standards is shown in Table 2.10.1. Fluorescence was linear with amount of cAMP between 0.375 to 15.0 pmol per injected sample (Fig. 2.10.1).

Table 2.10.1 Fluorescence peak area of standard etheno-cAMP (ε-cAMP)

Standard ε-cAMP (pmol)	peak area
0	0
0.375	6249
0.75	7130
1.5	14603
7.5	75988
15.0	142865

Fig. 2.10.1 ε-cAMP standard curve for cAMP estimation (data were derived from the Table 2.10.1, above). Preparation of standards and unknown samples were described in 10.c. Quantitation of cAMP was based on measuring peak area of the sample and comparing with standards.



RESULTS

SECTION III POSITIVE SELECTION OF cAMP REQUIRING CDC MUTANTS

Alternatives to genetic analysis of biological processes include application of chemicals with well defined inhibitory effects. Specific inhibitors whose mode of action are known can be valuable in establishing the role of reactions in cellular physiology. However metabolic inhibitors often have side effects that may complicate the interpretation of their effects and in addition there are relatively few such inhibitors compared with the number of biological processes. An alternative approach that, in principle, can allow the modification of any enzyme-catalysed process, is to obtain mutations that affect the enzyme. Therefore in the present study, mutations that were sensitive to the presence or absence of cAMP in their progress through the cell division cycle were sought. A positive selection screen was devised in which cells were initially selected for resistance to the known phosphodiesterase inhibitor, caffeine (Butcher and Sutherland, 1962; Sutherland, 1972). These were further investigated to identify any that were blocked in cell division but could be rescued by the addition of cAMP. Mutants were selected that could grow normally at a permissive temperature (21°C) in the presence of the phosphodiesterase inhibitor, caffeine and continue to grow at a non-permissive temperature (33°C) although unable to divide unless supplemented with cAMP.

Synchronous culture for mutagenesis

Synchronous division of *C. reinhardtii* was induced in minimum medium by alternation of light and dark periods (Bernstein, 1960, 1968; Kates and Jones, 1964a). Synchrony was monitored by following the gradual increase in average cell size through the cell cycle up to the point of daughter cell formation when there was a synchronous increase in cell number and decline in average cell volume. The initial mean cell volume (MCV) of normal wild type cells at the beginning of G1 phase was approximately 80 fl. Fig. 3.1 illustrates the cell size frequency profiles of the population as the cycle progressed. Cell size increased to an average of 1400 fl by 16 h but by 18 h it had declined due to the synchronous formation of daughter cells which resulted in a sevenfold increase in cell number relative to the starting cell number (Fig. 3.2). This increase in number indicates that most cells attained three commitments each to successive doubling of DNA and cell number (John, 1984), and thus the predominant number of offspring in these growth conditions was eight.

Nuclear DNA staining by mithramycin (Table 3.1) showed that cells that had completed mitosis began to appear between 13 to 14 hours as bi-nucleates. Second and third rounds of mitosis occurred at 15 to 16 h and by 18 h, eight daughter cells were formed within each mother cell wall. Daughter release was almost complete by 20 h and was completed before the beginning of the next period of illumination at 24 h.

The strategy for mutagenesis that was adopted was to select a time at which most cells were carrying out DNA replication, because it has been established that eukaryotic DNA is particularly sensitive to mutagenesis then (Riddle and Hsie, 1978). It was further attempted to select a time that would avoid mutagenesis in the early rounds of replication because early mutation would allow duplication of mutations by subsequent DNA replication and the risk of duplicate isolation of the same mutation. Therefore mutagenesis was not performed until nuclear staining of cells revealed that the first mitosis had occurred in at least half of the population and therefore later rounds of DNA replication were in progress. Under the conditions illustrated in Table 3.1, 13.5 h was selected as suitable for EMS mutagenesis.

EMS concentration and the percentage of caffeine resistant mutants

The objective of these tests was to observe the effect of EMS concentration on the yield of resistant colonies. EMS concentrations of 100 mM and 140 mM, which gave 80% and 30% survival respectively in an earlier test (Methods 5.a), were selected. A selective inhibitor, caffeine, which is known as cyclic nucleotide phosphodiesterase inhibitor in animal cells (Butcher and Sutherland, 1962 and Sutherland, 1972) as well as in *Chlamydomonas* cells (Amrhein and Filner, 1973) was used. Synchronously dividing cells at 13.5 h were mutagenized then grown for a period in rich medium (LSHSMAYPP) to dilute out the wild-type gene products and allow the expression of newly induced mutations that might confer resistance before exposure to caffeine.

The percentage of viable cells that were caffeine resistant was 0.078% after mutagenesis with 140 mM EMS and 0.043% after 100 mM EMS (Table 3.2). The lower concentration gave a lower percentage of mutants among survivors but the yield was offset by the higher numbers of survivors. Therefore both concentrations were considered to be suitable.

Isolation of temperature-sensitive (ts) caffeine resistant mutants

The caffeine resistant mutants that were obtained by mutagenesis with EMS at 100 mM and 140 mM were further screened to identify colonies that possessed thermolabile proteins that were essential for cell division. It was considered that these mutants would carry out normal vegetative cell division at 21°C but at 33°C would not divide although continuing to grow at this restrictive temperature therefore producing unusually large cells.

The isolation of these mutants was carried out by replica plating the caffeine resistant colonies to plates containing no inhibitor and then incubating at 21°C and 33°C. Non temperature-sensitive caffeine resistant mutants formed macroscopic colonies at 33°C whereas temperature-sensitive mutants produced microcolonies or no colony at all. The potential temperature-sensitive colonies were retested on solid agar by streaking cells thinly on TAPYPP agar and incubating at 33°C. Cells were inspected by microscopy to detect division block and cell enlargement.

A total of 4532 clones with caffeine resistance properties was obtained using EMS at 100 mM. Of these a total of 404 clones, or approximately 8.9%, were found to be temperature sensitive colonies on agar plates. The other treatment which used EMS at 140 mM gave a the total of 772 temperature-sensitive colonies (7.8%) from a total of 9933 caffeine resistant clones. Even though 140 mM EMS gave rise to the highest number of survivors that were caffeine- resistant, both concentrations gave approximately the same number of the temperature-sensitive colonies on agar (Table 3.3). However, further tests for temperature-sensitive cell division colonies in liquid medium showed that only 79 colonies or 6.7% of the total 1176 temperature-sensitive caffeine resistant colonies satisfied the criterion of being blocked in division in liquid. Clearly in this photosynthetic organism, which occurs naturally in liquid, illumination on the surface of agar presents a more hostile environment in which many proteins made imperfect by mutagenesis are unable to sustain division although they can in the milder conditions of liquid culture. Among the potential temperature-sensitive colonies selected on solid agar plates, only a few colonies had mutations in genes that are involved in the cell division cycle rendering their products conditionally unable to support cell division. The final yield was 0.00028% of individual cells initially exposed to EMS. Clearly an incidence of caffeine resistant cdc mutants of 1 per 3.5×10^5 treated individuals is practical for use with *Chlamydomonas* but

would probably be difficult for higher plant cells.

Test for cAMP or db-cAMP requirement

To isolate possible ts cAMP-requiring cdc mutants, the 79 ts-cdc in liquid-caffeine-resistant mutants were grown in medium with and without the supplementation of cAMP or dibutyryl cAMP (db-cAMP) with control tests in each case using 5'-AMP or butyrate respectively. Clones were assessed by counting final cell numbers in supplemented compared with unsupplemented medium. The wild-type strain cc-125^{+} was also tested for possible effects of cAMP supplementation in the concentration range that was used to screen mutants (Fig. 3.3). Neither cAMP nor db-cAMP in the concentration range 0.5 mM-5 mM showed any significant effect on wild-type cell number increase.

Four mutants were identified as responding specifically to cAMP by this screen (mutants 92, 194, 195, and 340). The defects caused by mutagenesis in each of these mutants were different. When mutant 92 was grown exponentially at 21°C and transferred to 33°C, there was only a small increase in cell number up to 48 h that may be attributed to cells that had passed the cAMP-requiring event being able to complete the cell cycle. Division block at 33°C was indicated because the cell number increase was only 1.8 fold compared with the 3.3 fold increase if temperature remained at 21°C and also in the division arrested culture at 33°C there was an increase in cell size (Fig. 3.5). Addition of cAMP or db-cAMP at the time of the temperature shift caused a marked increase in cell numbers (Fig. 3.4a). Cyclic AMP concentration was effective at 1mM, whereas 5'-AMP was ineffective. Dibutyryl cAMP showed the same effect as cAMP but at the lower concentration of 0.5 mM. The increase in cell number in the culture which was supplemented with cAMP or db-cAMP ceased after 48 h of incubation at 33°C. It would be interesting to test whether the cAMP had been degraded by then and whether further addition of cAMP would again restore the cell division. These results indicated that exogenous cAMP sustained cell cycle progression at 33°C.

Similar results were obtained from mutant 340 in that division of untreated cells was arrested and large cell sizes were observed at the non-permissive temperature while at the permissive temperature they could grow and divide normally (Fig. 3.4b and 3.6). The difference between mutant 92 and 340 was that mutant 340 was not able to mate. They were able to agglutinate but fusion did not occur. This observation is consistent with a

role for cAMP in the mating mechanism. There are reports which indicate increases in cAMP levels at the beginning of agglutination in *C. eugametos* (Pijst, et al. 1984b) before other events in the fusion process, which suggest that cAMP may be a primary signal triggering the events. The same phenomenon was demonstrated in *C. reinhartdii* (Pasquale and Goodenough, 1987). Furthermore an addition of exogenous cAMP increased the mating efficiency (Greenwood, 1973). Similar effects on mating have been reported to follow addition of the cAMP analogue, db-cAMP and the phosphodiesterase inhibitor, isobutyl methylxanthine (Pasquale and Goodenough, 1987).

The mutant 340 had lost its cAMP-requiring property after the first subcultures. Even though it continued to exhibit the arrested phenotype on solid agar plates, it failed to arrest in liquid medium at 33°C. The defect in mating made it impossible to study the mutated gene which involved cAMP and that affected cell division processes.

The response of mutants 194 and 195 to cAMP and db-cAMP was different from mutants 92 and 340. When first isolated mutants 194 and 195 were not completely blocked in cell division at 33°C, however they increased in cell size (Fig. 3.7) indicating that division did not keep up with growth and when supplemented with cAMP or db-cAMP increase in cell number was stimulated. Strain 194 was partially stimulated only by cAMP at 1 mM after 48 h (Fig. 3.4c) and strain 195 was partially stimulated only by db-cAMP at 0.5 mM (Fig. 3.4d). Their arrest and rescue by supplementation improved after back crossing to wild-type to remove extraneous mutations in other genes that were present after EMS treatment (section VI).

Fig. 3.1 Profile of cell size frequency during synchronous culture of *C. reinhardtii*

Wild-type (cc-125^{+}) cells were synchronized by alternate light and dark periods (14 h : 10 h) in LSHSMA medium. The culture was aerated continuously with filtered steriled humidified air which contained 0.5% (v/v) CO_2. The air was delivered at a rate of 1 l of air per min and 10 ml CO_2 per min per 2 l of culture. The culture was illuminated by warm white fluorescent lights at 200 $\mu Em^{-2}s^{-1}$ of PAR in the range of 400-700 nm. Cell numbers and cell sizes were measured by Coulter Counter Model ZB, connected to Coulter Channelyzer C-250 and a printer (EPSON). The system was equipped with a 80 μm orifice and calibrated with latex particles of 15 μm in diameter. Samples were taken at 0, 4, 8, 12, 14, 16, 18 and 24 h and fixed with 1% final concentration formaldehyde.

3.1

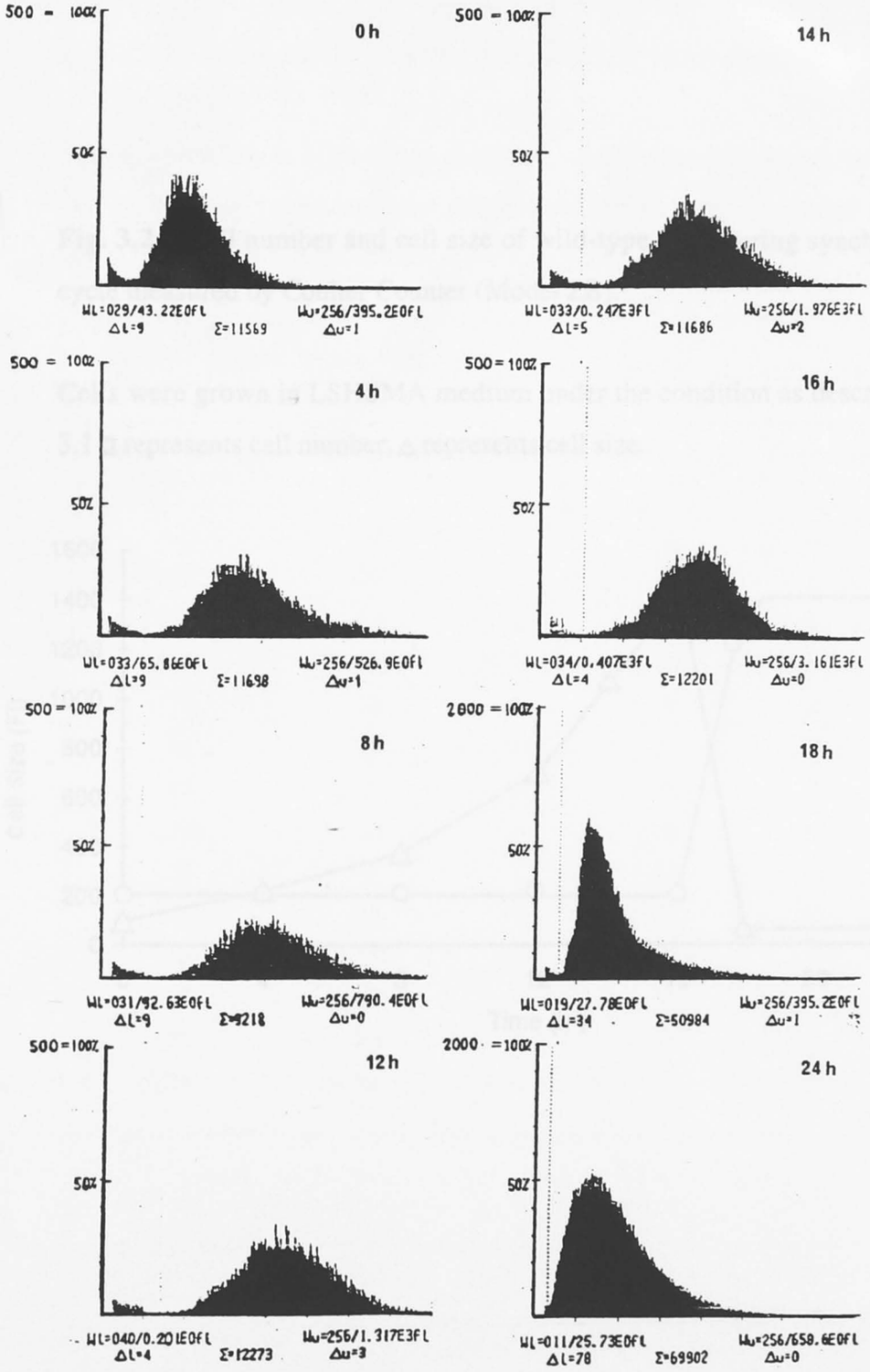

500 = 100%
50%
0 h
Wl=029/43.22E0fl
Δl=9
Σ=11569
Wu=256/395.2E0fl
Δu=1
14 h
Wl=033/0.247E3fl
Δl=5
Σ=11686
Wu=256/1.976E3fl
Δu=2
4 h
Wl=033/65.86E0fl
Δl=9
Σ=11698
Wu=256/526.9E0fl
Δu=1
16 h
Wl=034/0.407E3fl
Δl=4
Σ=12201
Wu=256/3.161E3fl
Δu=0
8 h
Wl=031/92.63E0fl
Δl=9
Σ=9218
Wu=256/790.4E0fl
Δu=0
2000 = 100%
18 h
Wl=019/27.78E0fl
ΔL=34
Σ=50984
Wu=256/395.2E0fl
Δu=1
12 h
Wl=040/0.201E0fl
Δl=4
Σ=12273
Wu=256/1.317E3fl
Δu=3
24 h
Wl=011/25.73E0fl
Δl=78
Σ=69902
Wu=256/658.6E0fl
Δu=0

Fig. 3.2 Cell number and cell size of wild-type cells during synchronous cell cycle measured by Coulter Counter (Model ZB).

Cells were grown in LSHSMA medium under the condition as described in Fig. 3.1 **O** represents cell number, **Δ** represents cell size.

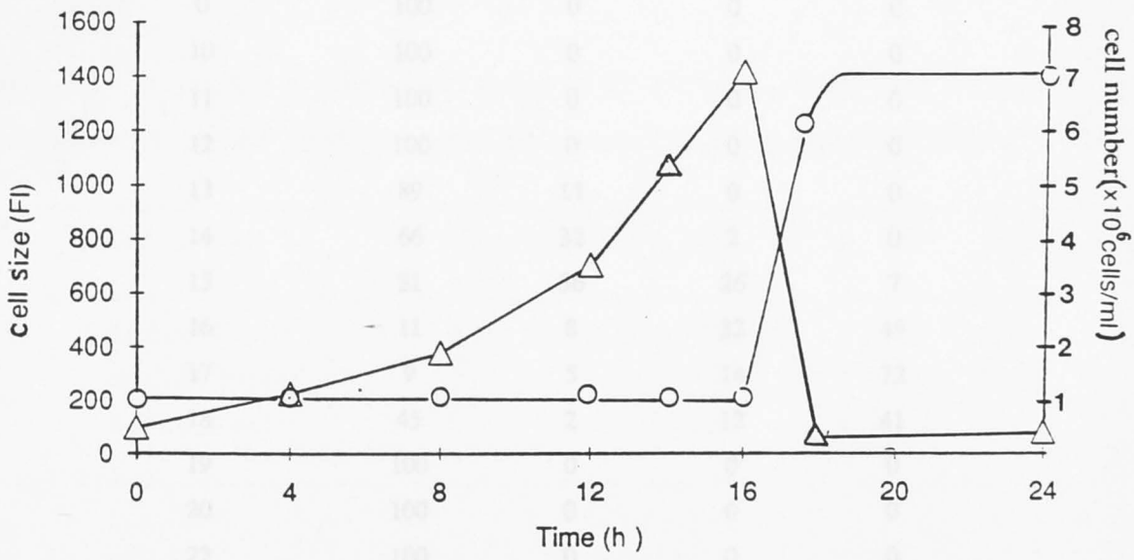


Table 3.1 Percentage of cells that contained 1, 2, 4 or 8 nuclei.

Samples were taken at the times indicated. Cells were dehydrated, permiabilised and decolorised with a series of ethanol treatments then rehydrated and stained with DNA fluorochrome, mithramycin (5 μg/ml).

nu indicates nuclear number per cell.

Sampling time (h)	%1 nu cells	%2 nu cells	%4 nu cells	%8 nu cells
0	100	0	0	0
10	100	0	0	0
11	100	0	0	0
12	100	0	0	0
13	89	11	0	0
14	66	32	2	0
15	31	36	26	7
16	11	8	32	49
17	9	5	14	72
18	45	2	12	41
19	100	0	0	0
20	100	0	0	0
22	100	0	0	0
24	100	0	0	0

Table 3.2 Percentage of viable cells that were caffeine resistant (caff.R) after mutagenesis with different concentrations of EMS

EMS (mM)	0	40	60	100	140	180
Total cells plated	$1x10^6$	$1x10^6$	$1x10^6$	$1x10^6$	$1x10^6$	$1x10^6$
Viable cells after EMS treated	$1x10^6$	$1x10^6$	$1x10^6$	$8.9x10^5$	$3.2x10^5$	$9.0x10^4$
Total caff.R	0	70	155	380	250	10
%viable cells that are caff.R	0	0.007	0.0155	0.043	0.078	0.011

Table 3.3 Percentage of caffeine resistant (caff.R) colonies obtained from mutagenized wild-type cells (cc-125^+) that were temperature-sensitive (ts) on agar plates at 33°C

EMS (mM)	0	100	140
Total caff.R	0	4532	9933
Total caff.R and ts	0	404	772
% of caff.R and ts	0	8.9	7.8

Fig. 3.3 Wild-type (cc-125^{+}) cells tested for the possible effects of cAMP and dibutyryl-cAMP (db-cAMP) on cell proliferation at 33°C.

Wild-type cells were grown in TAPYPP medium at 33°C without supplementation (○); with supplementation of 5 mM cAMP (◀); with supplementation of 5 mM of 5'AMP (▷); with the supplementation of 5 mM db-cAMP (◆); and with supplementation of 5 mM butyrate (◇). Samples were taken at every 24 h and fixed with 0.2 % final concentration of formaldehyde. Cell numbers were counted manually using the haemocytometer.

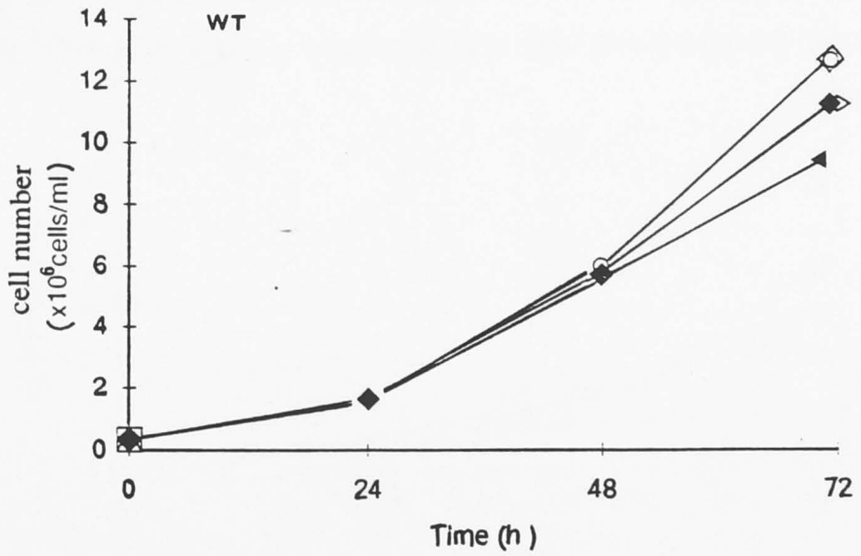


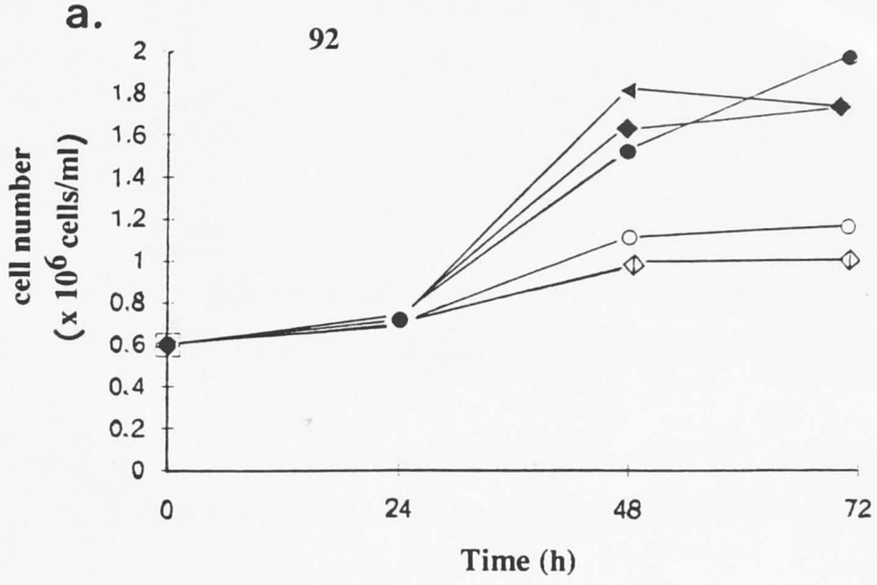
Fig. 3.4 cAMP and db-cAMP supplementation test in ts-caffeine resistant cdc mutants.

a. Mutant 92 grown in TAPYPP medium without supplementation at permissive temperature, 21°C (●) and at nonpermissive temperature (○) at 33°C. Cells were cultured in TAPYPP medium at 33°C in the supplementation of (◀) 1 mM cAMP, (▷) 1 mM 5'AMP, (◆) 0.5 mM db-cAMP and (◇) 0.5 mM butyrate. Sample were taken at every 24 h and fixed in 0.2% final concentration of formaldehyde. Cell numbers were counted manually using a haemocytometer.

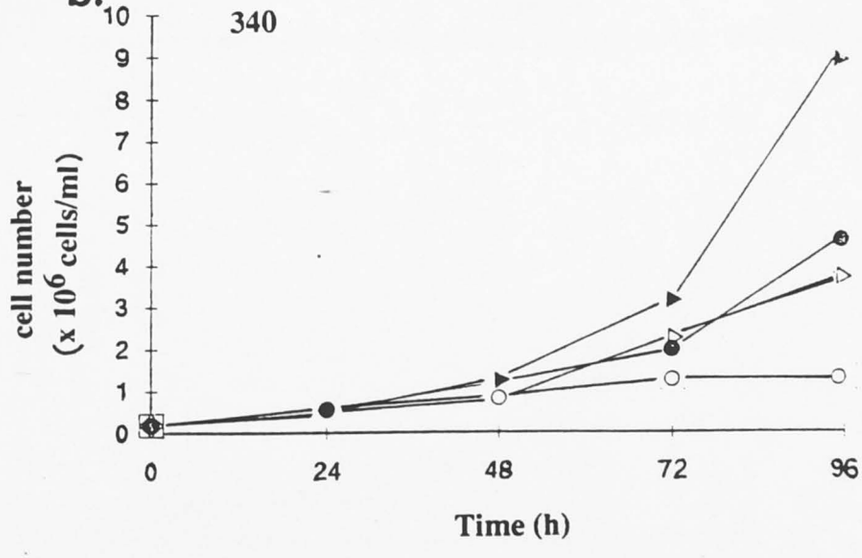
b. Mutant 340 grown in TAPYPP medium without supplementation at 21°C (●) and 33°C (○). (▶) cell cultured with the supplementation of 4.5 mM cAMP, (▷) 4.5 mM 5'AMP.

3.4

a.


92
cell number
(x 10^6 cells/ml)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
72
Time (h)

b.


340
cell number
(x 10^6 cells/ml)
10
9
8
7
6
5
4
3
2
1
0
0
24
48
72
96
Time (h)

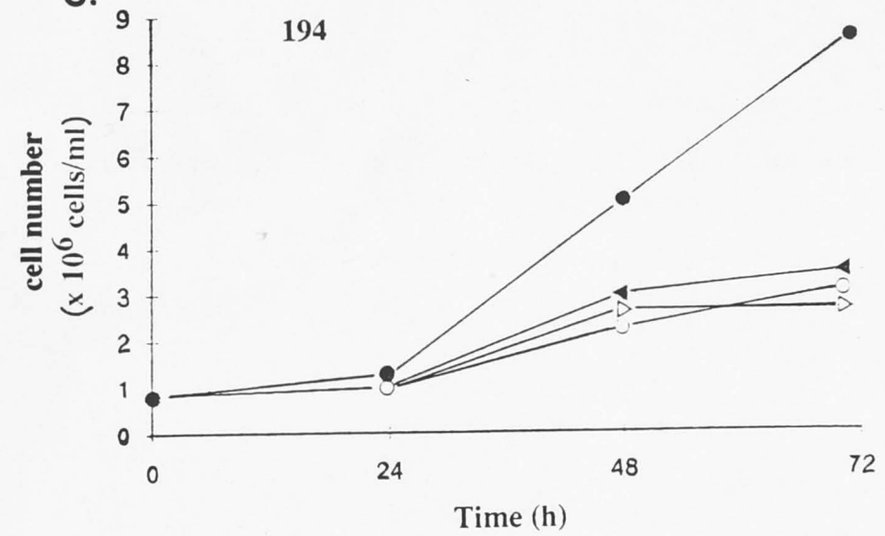
c. Mutant 194 grown in TAPYPP medium without supplementation at 21°C (●) and 33°C (○). (◀) cells cultured in the presence of 1 mM cAMP, (▷) 1 mM AMP.

d. Mutant 195 grown in TAPYPP medium without the supplementation at 21°C (●) and 33°C (○). (◆) cells were cultured in the presence of 0.5 mM db-cAMP, (◇) 0.5 mM butyrate.

The improvement of response to supplementation of cAMP or db-cAMP of mutants 194 and 195 after repeated back crossing with wild-type are shown in section VI.

3.4

c.


194
cell number
(x 10^6 cells/ml)
9
8
7
6
5
4
3
2
1
0
0
24
48
72
Time (h)

d.

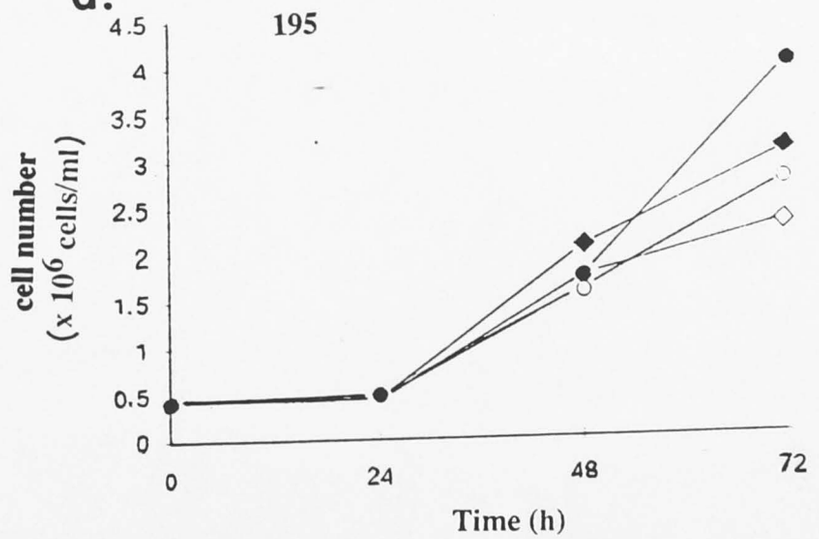

195
cell number
(x 10^6 cells/ml)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
24
48
72
Time (h)

Fig. 3.5 Original isolates of mutant 92 tested for division arrest and cAMP and db-cAMP response

Cells were cultured in TAPYPP medium supplemented with 1 mM cAMP, 1 mM AMP, 1 mM db-cAMP or 1 mM butyrate. Cells were taken after 48 h incubation, fixed in ethanol, dehydrated, rehydrated and stained with 5 µg/ml mithramycin.

a. cells cultured at 21°C

b. cells cultured at 33°C.

c. cells cultured at 33°C with the supplementation of cAMP 1 mM

d. cells cultured at 33°C with the supplementation of db-cAMP 0.5 mM

Left panel represents cells observed by phase-contrast and right panel represents DNA stained cells observed by fluorescence.

3.5

ts cdc 92

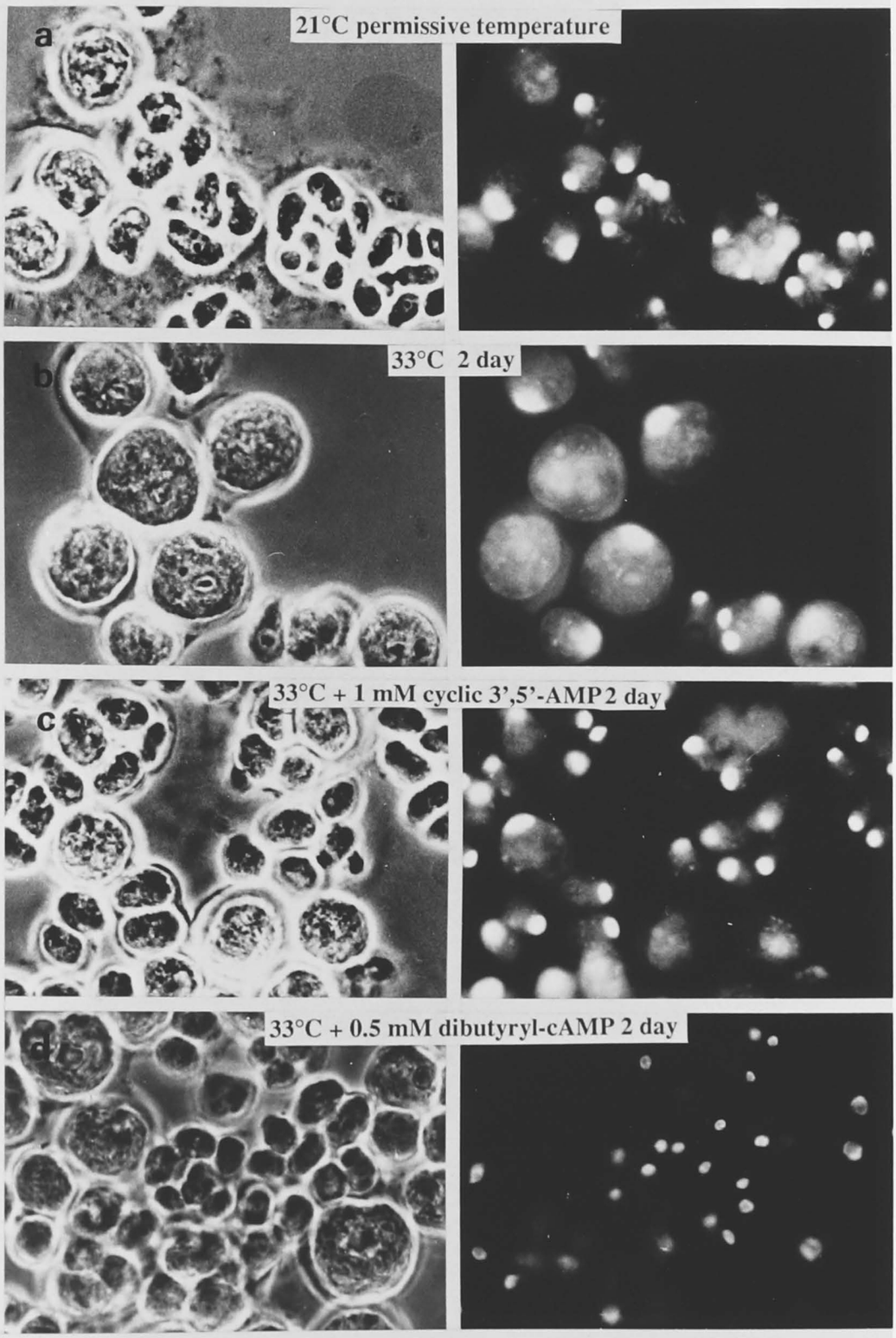

a
21°C permissive temperature
b
33°C 2 day
c
33°C + 1 mM cyclic 3',5'-AMP 2 day
d
33°C + 0.5 mM dibutyryl-cAMP 2 day
10 µm

Fig. 3.6 Original isolates of mutant 340 tested for cAMP and db-cAMP response.

Cells were cultured in TAPYPP medium with and without supplementation of cAMP and db-cAMP. Cells were taken after 48 and 120 h, dehydrated in ethanol, rehydrated and stained with 5 µg/ml mithramycin.

a. cells cultured at 21°C

b. cells cultured at 33°C after 48 h

c. cells cultured at 33°C after 120 h

d. cells cultured at 33°C with the supplementation of 4.5 mM cAMP after 120 h

Left panel represents phase contrast, right panel represents DNA stained cells observed by fluorescence.

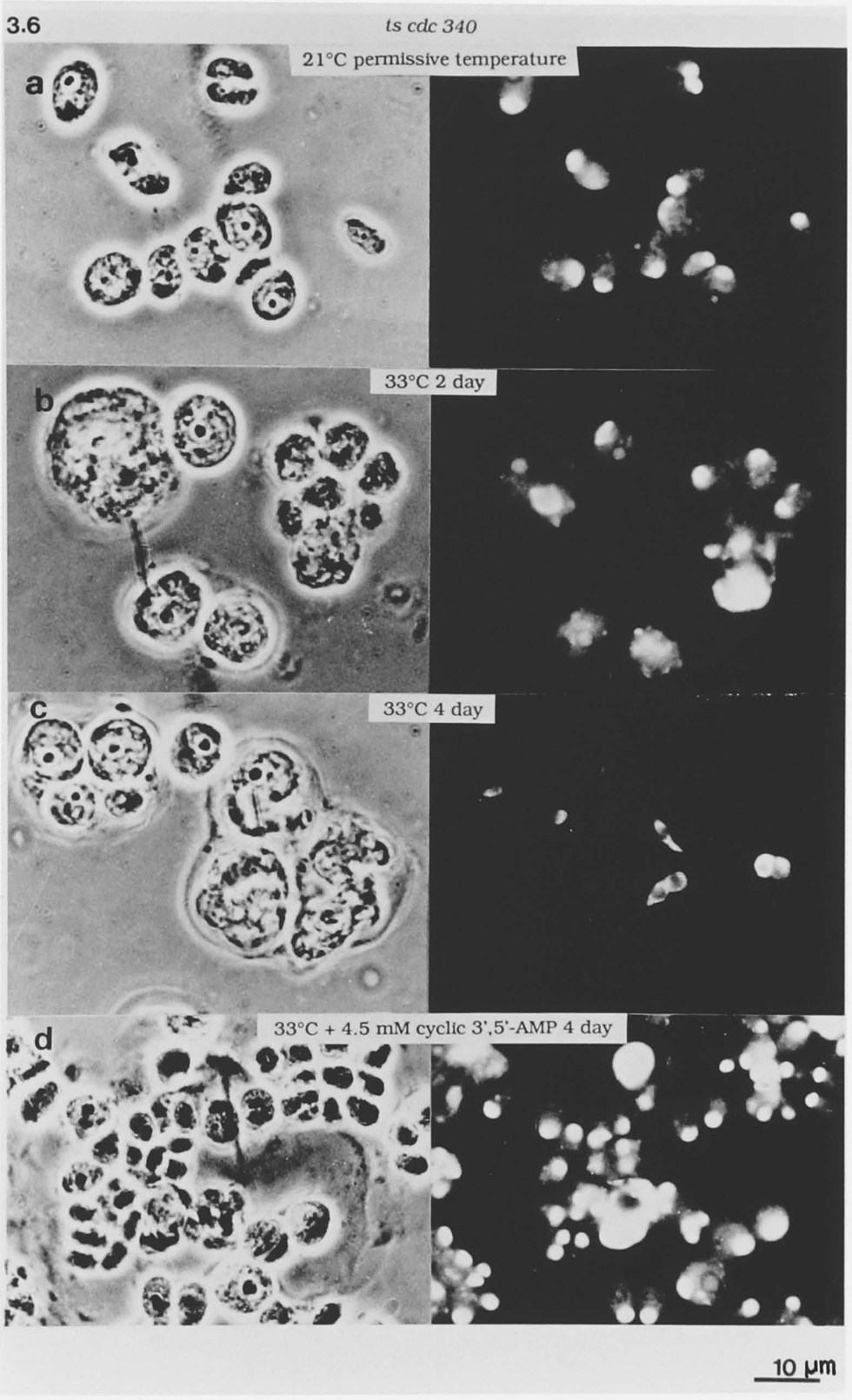
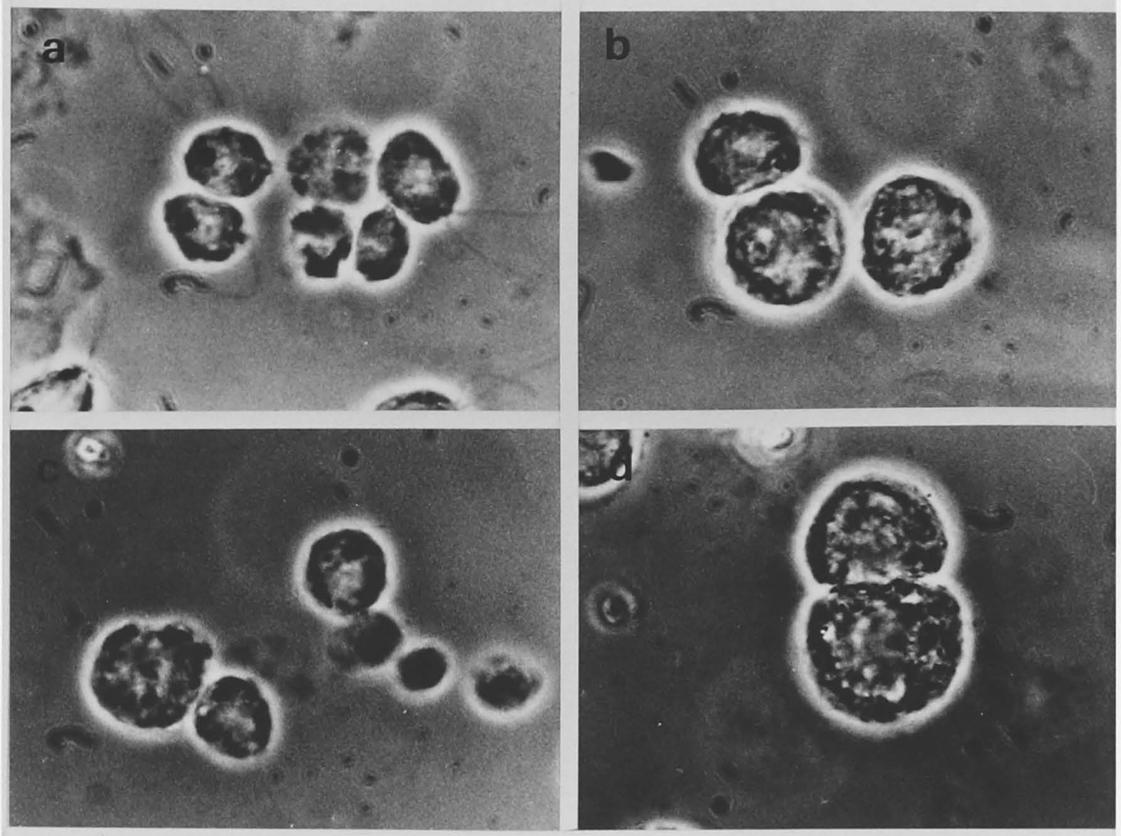

3.6
ts cdc 340
21°C permissive temperature
a
33°C 2 day
b
33°C 4 day
c
33°C + 4.5 mM cyclic 3',5'-AMP 4 day
d
10 µm

Fig. 3.7 Original isolates of mutants 194 and 195 cultured in TAPYPP medium;

a and b, mutant 194 cultured at 21°C and 33°C respectively after 48 h

c and d, mutant 195 cultured at 21°C and 33°C respectively after 48 h

3.7



SECTION IV. DNA CONTENTS OF ts cAMP-REQUIRING CDC MUTANTS

To determine the phase of the cell cycle at which the cAMP-requiring mutants arrest, the nuclear DNA content of each mutant was measured under culture conditions that permitted and restricted cell division. Because this information is crucial to the correct identification of the arresting phase, reliance was not placed on a single DNA assay method.

The first type of method was biochemical and employed two different chemical reactions. Nucleic acid was extracted and deoxyribose present in DNA was reacted with diphenylamine for a colorimetric assay (modified from Hopkins et al., 1972) or, by a modified fluorometric method, with diaminobenzoic acid dihydrochloride, DABA.2HCl (modified from Lien and Knutsen, 1976). The DNA content per cell obtained from these chemical methods was calculated by dividing the deoxy-ribose detected by the total cell number. This type of estimation has been widely used to determine the time of DNA replication in synchronous cultures of *Chlamydomonas* and is successful for that purpose since the increase in DNA is often four or eight fold, however there is interference from other cell constituents. In assays of division-arrested mutants, which attain large cell size, this interference may be greater. Therefore an increased signal may wrongly indicate replication of DNA in the arrested cells.

The second method estimated DNA from individual nuclei by using fluorescent nuclear stains. DNA levels were calculated in whole nuclei, and expressed as a percentage of fluorescence in standard chicken red blood cell nuclei. The fluorescence measurements were made using a MRC-500 laser scanning microscope.

DNA assay by chemical reactions

Two different chemical reactions with DNA were compared. The first reaction was the diphenylamine colour reaction, which has been widely used for determining deoxyribonucleic acid because of its high sensitivity and relatively low blank. The colour results from the amine residue of diphenylamine reacting with sugar residues of DNA. Acetaldehyde or other aldehydes are used to potentiate colour development (Burton, 1955), however the nature of the colour compounds formed in the diphenylamine reaction is unknown. The second reaction that was employed during this research is based on the measurement of the fluorescent product from the reaction between 3',5'-diaminobenzoic

acid dihydrochloride (DABA.2HCl) and deoxyribose. Because of potential interference in this reaction from compounds with low molecular weight such as pigments, carbohydrates or free nucleotides it was necessary that these compounds be removed with the minimum loss of nucleic acid. According to Lien and Knutsen (1976), a pre-extracted with cold trichloroacetic acid (TCA) followed by ethanol-ether (3:1) extraction was found to be effective. In the present study a further improvement in the extraction was made by incubation with 1 N NaOH at room temperature according to Hopkins et al. (1972).

To investigate if cell size interferes with the chemical estimation of DNA content samples were taken from synchronous wild-type cells at early G1 (0 h and 24 h) when cells were still small and DNA has not been replicated, in late G1 (8 h) when cells have increased in size but DNA are still 1C, and at 12 h when there is a mixture of cells in G1, S and G2. As shown in Table 4.1 the cellular DNA content remained approximately constant for the first 8 h, and then increased two fold between 8 and 12 h. At 24 h, division had been completed and the cellular DNA content dropped to a level similar to that observed during the first 8 h. These estimates indicate that the DNA content of a vegetative cell from synchronous culture before replication is 0.22-0.25 pg per cell, which is close to earlier reports of 0.123 pg per cell by Chiang and Sueoka (1967), 0.2 pg per cell by Lien and Knutsen (1976) and 0.2 pg per cell by Lemieux (1980).

The similarity of the results that were obtained from wild-type cells using two different chemical assay methods suggested that the assays were potentially reliable for use with normal cells. However there is a particular problem concerning their use with mutant cells and this is the large size attained by cells that continue to grow while specifically blocked in cell division. There is a potential hazard that the larger amounts of cell mass will increasingly introduce false positive reactions. To test the effect of different amounts of cell material on the performance of the chemical assays a range of cell numbers was taken for assay in early G1 (0 and 24 h), late G1 (8 h) and G1, S and G2 (12 h). There was no consistency of DNA per cell over the range of cell number assayed (10-100 x 10^6 cells). Increasing numbers of cells taken for each sample and increasing cell size caused an apparent increase in DNA per cell in the reaction with DABA.2HCl while decreasing the apparent DNA per cell indicated by diphenylamine reaction (Table 4.2). However the apparent DNA content of cells at 0 h and 8 h when assayed by both DABA.2HCl and diphenylamine was similar when DNA was extracted from total 100 x 10^6 cells in each case

and thus there is not complete disagreement. However it is clear that chemical reactions of DABA.2HCl and diphenylamine are subject to interference from other cell components. This is tolerable in wild-type cells that do not become unusually large, but for the particular purpose of diagnosing whether blocked cdc cells had entered S phase a problem clearly exists for use of these methods.

When the chemical assays for DNA were applied to the mutants isolated here, both mutant cells and wild-type controls were grown asynchronously at 21°C until the cultures reached exponential phase, then portions were shifted to 33°C. After 24 h, samples were taken for DNA estimation. The result, as shown in Table 4.3, indicated that average values of DNA content per vegetative wild-type cell from asynchronous cultures were higher than the value obtained in G1 phase from the synchronous cultures illustrated previously (Table 4.1). This was because the asynchronous culture contained cells from all stages of the cell cycle including S and G2 nuclei that have higher DNA contents. Chemical assays indicated that mutant 92 cells were apparently arrested in G2 phase in the absence of cAMP at 33°C since DNA content was double that of G1 cells. This estimate was plausible although inflation by interfering chemicals was suspected. Reproducible estimates of DNA content in 194 and 195 could not be obtained from these chemical methods. The DNA content of mutant 194 after arrested at 33°C for 24 h was more a than 5 fold higher than the DNA content of non arrested cells at 21°C. DNA contents of mutant 195 cultured at both 21°C and 33°C gave inconsistent results by DABA.2HCl reaction and furthermore these values did not correlate with the values obtained from the diphenylamine reaction. The inconsistency and the unlikely high DNA contents could be introduced by unknown substances that were abundant in arrested cells and in addition there may also have been a contribution from extranuclear DNA, such as that of chloroplasts, that might be present in abnormal numbers in the large arrested cells.

To avoid chemical interference and contribution from organelle DNA a method for the specific estimation of nuclear DNA was developed.

Table 4.1 Average DNA content of wild-type cells at early G1 (0 and 24 h), late G1 (8 h) and G1, S and G2 period (12 h), estimated by diaminobenzoic acid dihydrochloride (DABA.2HCl) reaction and diphenylamine reaction

Sampling period (h)	Total cells $x10^6$	DABA.2HCl (DNA pg/cell)	Diphenylamine (DNA pg/cell)
0	100	0.16	0.18
8	120	0.25	0.24
12	100	0.44	0.51
24	146	0.21	0.2

Cells were harvested from synchronous culture at 0, 8, 12 and 24 h of the cycle and treated with 0.5 N NaOH overnight. The suspension was neutralized with 0.5 N HCl. The cells were pelleted and then extracted with ethanol:ether (3:1). DNA was extracted by treating cells with 0.5 N perchloric acid (PCA) at 90°C for 20 min and the supernatant was used for assay of DNA contents.

For the diphenylamine reaction, 1 ml of cell extract was mixed with 2 ml Burton's reagent for 14-20 h at 30°C. Absorbance was measured at 600 nm.

For DABA.2HCl, 0.2 ml of cell extract was mixed with 0.3 ml 1 N NaOH and 0.2 ml of 20% (w/w) DABA.2HCl then incubated at 60°C for 40 min. Reaction was stopped by adding 4 ml of 0.6 N PCA. Fluorescence was measured by a fluorescence spectrophotometer Hitachi F.3000 (Hitachi Ltd. Tokyo, Japan) using an excitation at 430 nm and emission at 530 nm.

Table 4.2 Average DNA content of wild-type cells taken from synchronous culture at early G1 (0 and 24 h), late G1 (8 h) and G1, S and G2 period (12 h). Total cells taken in each case covered the range from 10-100 x 10^6 cells, to observe interference of the DABA.2HCl and diphenylamine reactions by increasing number of cells taken for each reaction. Possible effects of increasing cell size were tested by taking samples at different phases of the cell cycle.

Sampling period (h)	Total cells x10^6	DABA.2HCl (DNA pg/cell)	Diphenylamine (DNA pg/cell)
0	10	0.037	0.35
	20	0.044	0.2
	40	0.041	0.087
	50	0.09	0.11
	60	0.66	0.1
	80	0.071	0.08
	100	0.16	0.16
8	10	0.049	0.45
	20	0.057	0.26
	40	0.08	0.18
	50	0.25	0.22
	60	0.104	0.16
	80	0.095	0.11
	100	0.25	0.28
12	10	0.059	0.55
	20	0.095	0.425
	40	0.098	0.22
	50	-	-
	60	0.128	0.19
	80	0.19	0.23
	100	-	-
24	10	0.042	0.38
	20	0.047	0.21
	40	0.058	0.13
	50	-	-
	60	0.069	0.10
	80	0.083	0.09
	100	-	-

Table 4.3 Average DNA content of asynchronous wild-type and mutant cells grown at 21°C and 33°C and assayed for DNA content by DABA.2HCl and diphenylamine reactions

Cell type	Culture temperature °C	DABA.2HCl (DNA pg/cell)	Diphenylamine (DNA pg/cell)
wild-type	21°	0.17	0.16
	33°	0.37	0.32
92	21°	0.48, 0.42	0.39
	33°	0.76, 0.92	0.42
194	21°	0.69, 0.30	0.34
	33°	3.54, 2.78	2.08
195	21°	0.27, 0.38	1.09
	33°	0.26, 0.958	2.22

Cells were grown at 21°C until the culture reached exponential phase. Then portions of culture were shifted to 33°C and cultured for 24 h. Cells were harvested and DNA contents were assayed as described in Table 4.1. Where two values of DNA content of cells at 33°C are shown, these result from duplicate samples, which showed the inconsistency of the method. It is evident that DNA values estimated from DABA.2HCl reaction are not correlated with DNA content obtained from diphenylamine reaction.

Quantitative fluorescence microscopy of nuclear DNA

A method was developed for estimation of nuclear DNA using a confocal microscope. The DNA-binding fluorochromes; propidium iodide (PI), mithramycin and chromomycin A3 (ch.A3), were examined for suitability in the measurement of *Chlamydomonas* DNA using the Confocal Laser Scanning microscope (LSM). This microscope allows the direct three dimensional reconstruction of biological specimens from planes of focus obtained with a moving mechanical stage and a shallow depth of focus field. When sequential sections in regularly spaced planes of focus were obtained, a three dimensional structure of the nucleus could be reconstructed and total DNA content could be calculated.

For estimation of DNA content in the *Chlamydomonas* nucleus the fluorescence intensity of the nucleus and the area of the nucleus in the plane of focus were measured in each optical section. The volume of each nuclear section was calculated using the spacing between the optical sections and area of the nucleus. From volume and intensity the fluorescence content of that section was calculated. The total fluorescence value of a nucleus was then obtained from the summation of fluorescence value from each consecutive section. The total nuclear DNA level of the measured *Chlamydomonas* cells was expressed as a percentage of the nuclear fluorescence value of chicken red blood cells (RBC), reported to contain 2.58 pg of DNA (Mirsky and Osawa, 1961), which were present on the same slide and were stained and measured in parallel. It is uncertain whether it is appropriate to subtract background fluorescence observed in the surrounding cytoplasm from the nucleus estimate, because the cytoplasmic signal may derive from components that are excluded from the nucleus. Therefore estimations were calculated both with and without background correction.

Consistency of nuclear DNA estimates tested with chicken RBC

Variation in fluorescence was observed with particular batches of stain and period of staining. Slides stained and stored at different times yielded different readings because of dilution and evaporation of the stored stain. It is valuable to have an internal reference to indicate any variation in staining procedures so that nuclear DNA contents of different cell samples can be compared. The reference must be stable, reproducible and easily distinguishable from the test cells with which it is mixed. Chicken RBC are terminally differentiated, homogeneous nondividing cells that have been used successfully

as reference in Fuelgen absorption microspectrophotometry (Rash, 1971) and flow microspectrophotometry (Hamilton, 1980) and were used in the present study.

The fluorescence values of the chicken RBC stained with PI are shown in Table 4.4 and the mean and standard deviation show the consistency of the estimations. Although chicken RBCs were used as an internal reference, they were not used as a calibration standard to estimate the absolute weight of DNA in the *Chlamydomonas* nucleus. This is because binding of each fluorochrome may be influenced by the base composition of the DNA in different organism. In particular chromomycin A3, like mithramycin, binds preferentially to G-C rich regions (Waring, 1971). However PI, which intercalates between bases in the DNA spiral (Hudson et al., 1969) may be less sensitive to the proportion of individual bases. Measurement of *Chlamydomonas* and RBC nuclei on the same cover slip in every case allowed the plant DNA levels to be expressed as a percentage of the chick internal standards and therefore permitted directly comparisons between different experiments.

Evaluation of DNA Fluorochromes

Three fluorochromes were tested for sensitivity in detecting the amount of nuclear DNA in *Chlamydomonas*. These stains were propidium iodide (PI), chromomycin A3 and mithramycin. Mithramycin and chromomycin A3 are closely related antibiotics. Both of them bind to DNA at G-C rich sites, both requires the presence of a metal ion such as Mg^{2+} and fluoresce yellow with DNA (excitation 430-450 nm, emission 585 nm) (reviewed by Harris, 1988). Propidium iodide is a nucleic acid intercalating fluorochrome that fluoresces bright red (excitation 520 nm, emission 620) (reviewed by Harris, 1988). Since PI binds not only to DNA but also double-stranded RNA, it was necessary to pretreat the sample with RNAse (Coleman,1981) and this was found to greatly reduce cytoplasmic fluorescence (Fig. 4.1 a and b). To test for specificity of the propidium iodide stain cells were treated with DNAse and RNAse before staining. No nuclear fluorescence could be detected in the cells treated with DNAse and RNAse (Fig. 4.1 c and d) indicating specificity of PI for DNA in the RNAse treated cells.

Comparison of PI, mithramycin and chromomycin A3 staining in synchronous wild-type cells.

To examine relative DNA contents detected by each stain, samples of synchronous wild type cells were taken at early G1 before DNA replication when cells were still small, in late G1 (8 h) when cells had increased in mass but DNA content had not increased and at 12 h when cells were present in G1, S and G2 phases. Each sample was pretreated with RNAse before being stained with PI, mithramycin or chromomycin A3. Fluorescence obtained from mithramycin was rather weak and showed a high level background interfering (Fig. 4.2b). Chromomycin A3 showed better fluorescence and lower background than that of mithramycin (Fig. 4.2c). Fluorescence obtained from propidium iodide stained cells was stronger than that of chromomycin A3 and mithramycin and had a low background (Fig. 4.2a). Propidium iodide and chromomycin A3 were further examined to establish the best fluorophore for this method. The fluorescence values of synchronous wild-type cells taken at early G1 (0 h), late G1 (8 h) and G1, S and G2 periods (12 h), stained with propidium iodide were distributed as shown in Fig. 4.3 and those of chromomycin A3 stain are shown in Fig. 4.4. To facilitate the comparison, mean values of each sample were calculated as shown in Table 4.5. Both stains showed similar distribution patterns. In the population of cells taken at 0 and 8 h, each nucleus would contain 1C amount of DNA typical of a G1 uninucleate cell. Relative DNA values of cells taken at 12 h were bimodal because cell populations taken at this hour included cells in G1 and G2 phase with some intervening cells in S phase. Using PI stain, the G1 DNA content estimated after background correction is around 5-6 units which predicted that cells at G2 would contain 12 units of DNA and the estimate in the 12 h sample showed the G1 values around 5 unit and the G2 value around 12 unit as estimated in 0 and 8 h samples. Similar correlation was obtained from the values estimated without background correction (Table 4.5). The relative DNA contents of cells stained with PI and chromomycin A3 were slightly different. Chromomycin A3 was considered less useful because although at 12 h the distribution was bimodal with peaks presumably of 1C at 11 units and 2C at 22 units estimated after background correction, the 1C amount at 12 h does not correlate with the 1C amount at 0 and 8 h, which are lower (Table 4.5). Similar correlations were observed from the chromomycin A3 values estimated without background correction (Table 4.5). In view of this bias and the decrease

of the 1C signal in the sample at 0 and 8 h and the lower intensity of the fluorescence with chromomycin A3, PI was selected as the most effective fluorochrome for measurement of *Chlamydomonas* nuclear DNA.

Using PI, the distributions of relative DNA values of cells incubated at 21°C and 33°C of each mutant were plotted separately as shown in Fig. 4.5. A G2 mutant which arrested in G2 phase (2C) at non-permissive temperature were used as a positive control of the method to confirm the detection of G1 (1C) DNA level of non-arrested cells and G2 (2C) level of cells arrested at non-permissive temperature (Fig. 4.5 g and h). The calculation for mean values and the estimated DNA ranges of each mutant are shown in Table 4.6.

Although 2C DNA content could be detected by this method as shown in the DNA content of G2 mutant cells arrested at non-permissive temperature, none of the mutant cells showed 2C DNA (Table 4.6). Relative DNA contents of the arrested cells of all three mutants corresponded to their haploid (1C) levels since the values from both incubation conditions cover the same range. Therefore these results indicated that the ts cAMP-requiring cdc mutants were arrested in the G1 phase of the cell cycle in the absence of cAMP or db-cAMP at 33°C.

Table 4.4 Consistency of the fluorescence values of chick red blood cell (RBC) stained with PI 2.5 μg/ml. The values were calculated both with and without background correction.

	RBC No.	Background correction	No Background correction
Sample 1	1	228358	232879
	2	231398	236066
	3	228019	232352
	4	193880	199790
	5	209552	215034
	6	210646	215356
	7	160308	166701
	8	180177	186865
	9	223271	229681
	10	245172	250582
	11	233439	238009
	12	231436	237009
	13	232464	237130
	14	218927	223263
	15	238985	244364
	n = 15	$\bar{x}$ = 217735 cv = 10.4	$\bar{x}$ = 223005 cv = 9.94
Sample 2	1	270198	275068
	2	285726	290602
	3	269196	275084
	4	278389	284354
	5	285412	290123
	6	263448	267410
	7	279571	283039
	n = 7	$\bar{x}$ = 275991 cv = 2.86	$\bar{x}$ = 280811 cv = 2.84

Fig. 4.1 Staining pattern of *C. reinhardtii* stained with PI (2.5 µg/ml) before and after RNAse treatment

Cells were fixed, dehydrated and decolourised in ethanol. After rehydration cells were either spread on coverslips carrying chick red blood cells (rbc) and then stained with PI 2.5 µg/ml for 3 h, or they were treated with enzyme as indicated below then spread on coverslips carrying RBC and stained with PI 2.5 µg/ml for 3 h.

a. Late G1 cells stained with PI without RNAse pretreatment. *Chlamydomonas* cells showed a high level of interfering background staining.

b. Late G1 cells stained with PI after RNAse pretreatment (1 mg/ml at 37°C for 30 min). *Chlamydomonas* cells showed no interfering background staining.

c. Early G1 cells stained with PI (2.5 µg/ml) after treatment with RNAse at 1 mg/ml at 37°C for 30 min.

d. Early G1 cells stained with PI (2.5 µg/ml) after treatment with both RNAse (1 mg/ml) and DNAse (1 mg/ml). RBC are stained but absence of fluorescence in *Chlamydomonas* cells indicates, by comparison with treatment (c), specificity of PI staining of DNA in RNAse treated cells.

rbc = chicken red blood cell
Chl = *Chlamydomonas* cell

4.1

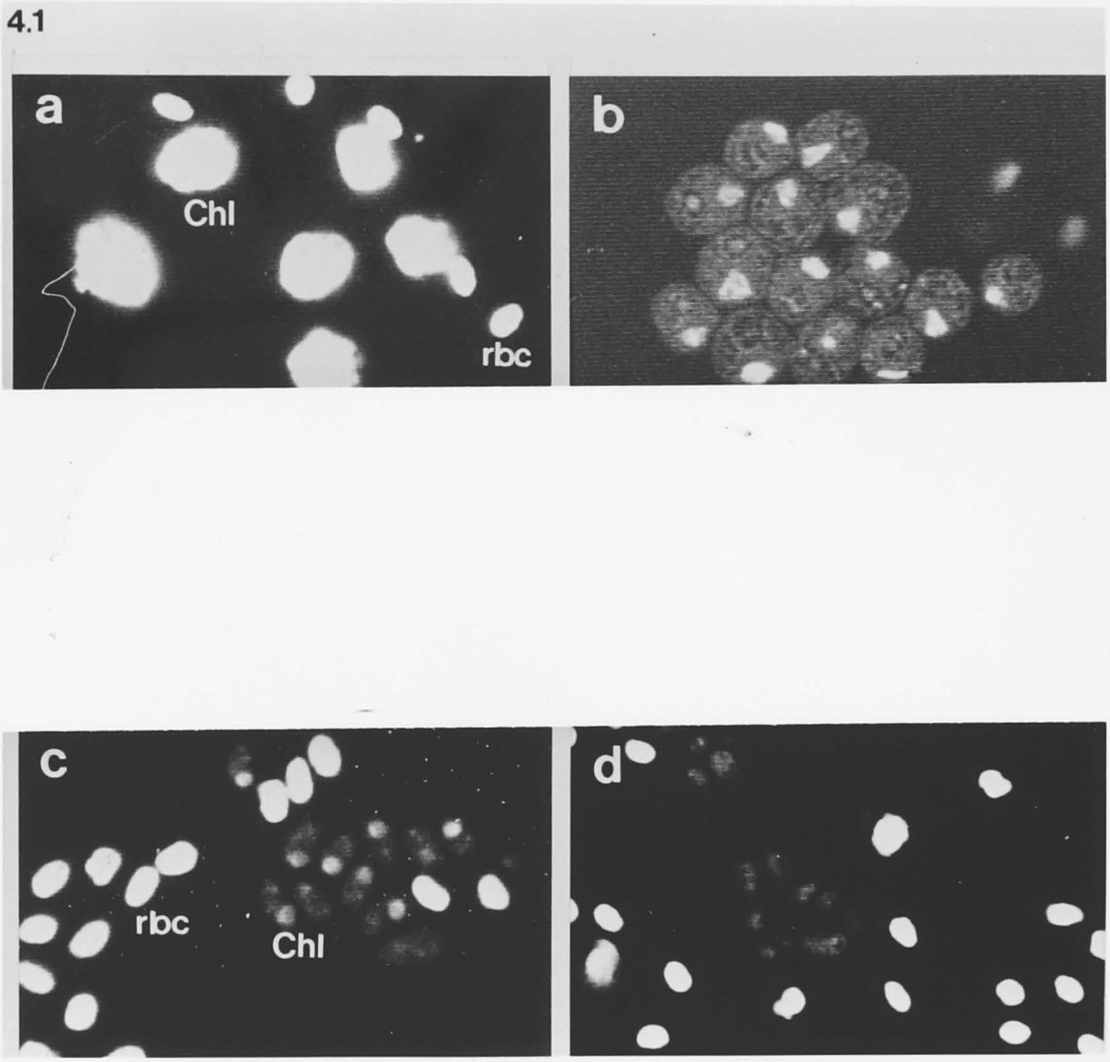


Fig. 4.2 Fluorescence of wild-type cells taken at 8 h from a synchronous culture in G1 phase stained with propidium iodide (PI), mithramycin or chromomycin A3 (ch.A3) observed using the confocal laser scanning microscope (MRC-500)

a. RNAse treated cells stained with 2.5 µg/ml PI (3 h). The stained cells showed strong fluorescence specific to the nucleus.

b. RNAse treated cells stained with 5 µg/ml mithramycin (3 h). The stained cells showed a high level of interfering background and weak nuclear fluorescence.

c. RNAse treated cells stained with 5 µg/ml chromomycin A3 (3 h). The stained cells showed no interfering background but weak nuclear fluorescence.

Scale bar = 10 µm

rbc = chicken red blood cell

Chl = *Chlamydomonas* cell

4.2

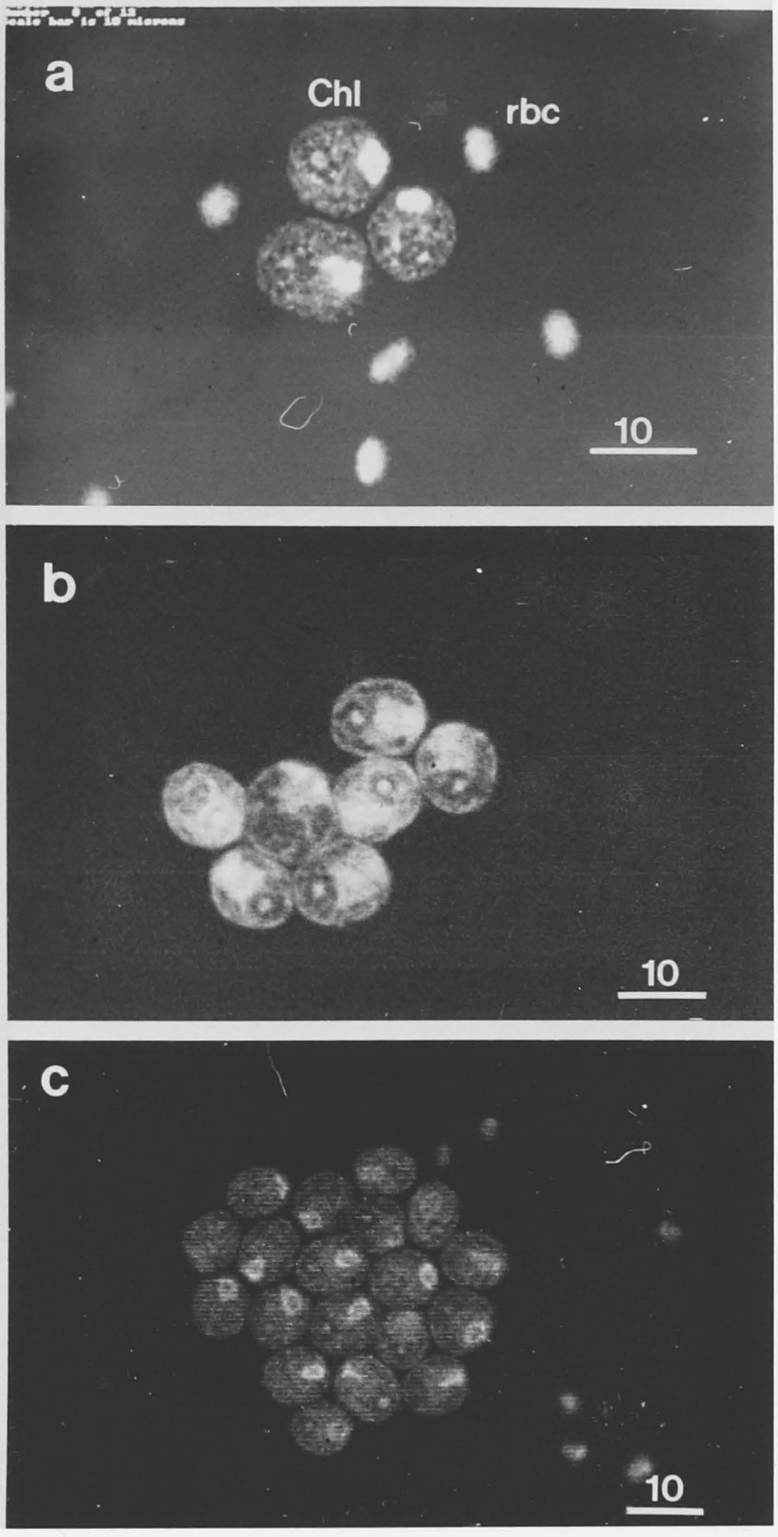


Fig. 4.3 Frequency of fluorescence values of nuclei in individual cells of wild-type taken from synchronous culture in early G1 (0 h) when cells were small and DNA had not been replicated (1C level), late G1 (8 h) when cells had increased in cell mass but DNA was still at 1C level and 12 h when there were cells in G1, S and G2 phase. This experiment shows that the method can differentiate DNA content in cells at G1, S and G2 phase. Stain was 2.5 μg/ml PI

Left panels represent values with background correction
Right panels represent values without background correction
x-axis represents frequency; y-axis represents percentage of the fluorescence of chicken red blood cells stained and quantified in parallel

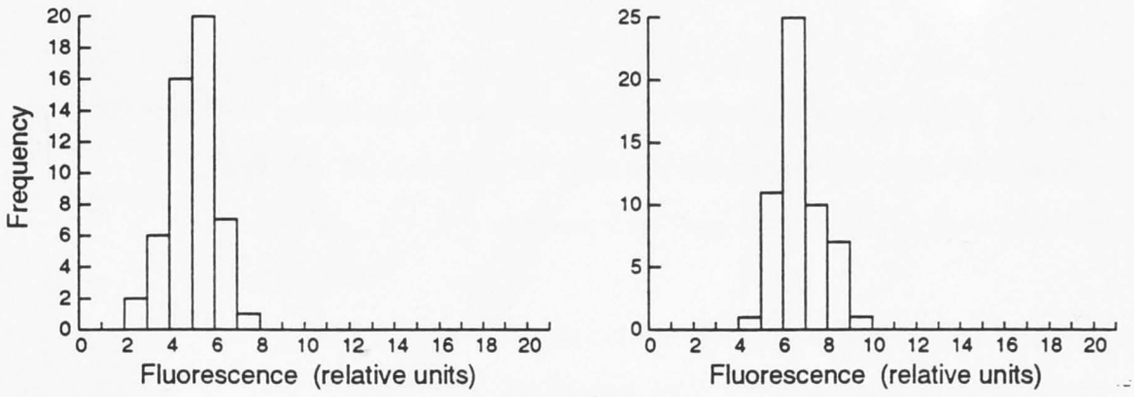
a and b within chromatograms heading " wt 12 h PI stain: G1 and G2" are frequency of individual nuclear fluorescence value measured from cells that were visually identified as having typical G2 nuclear appearance, i.e., cells with elongated nucleus.

4.3

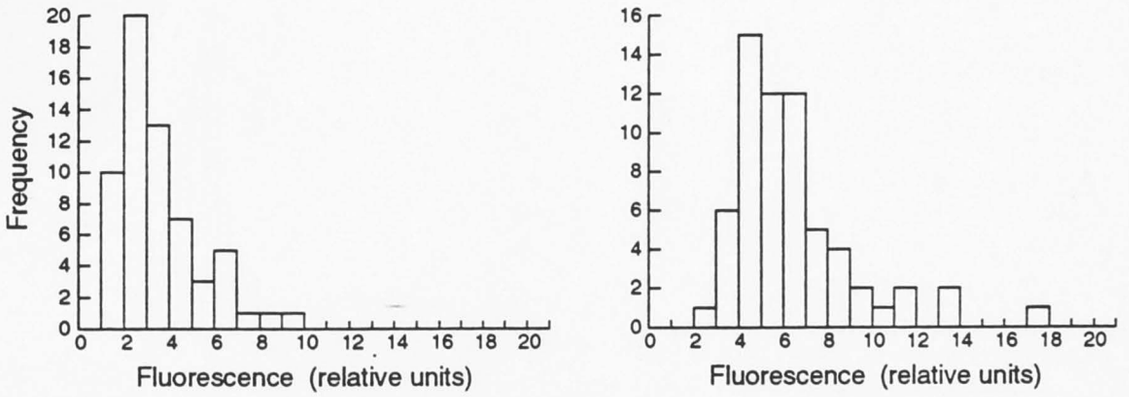
Background correction made

No background correction

wt 0 h PI stain : early G1



wt 8 h PI stain : late G1



wt 12 h PI stain : G1 and G2

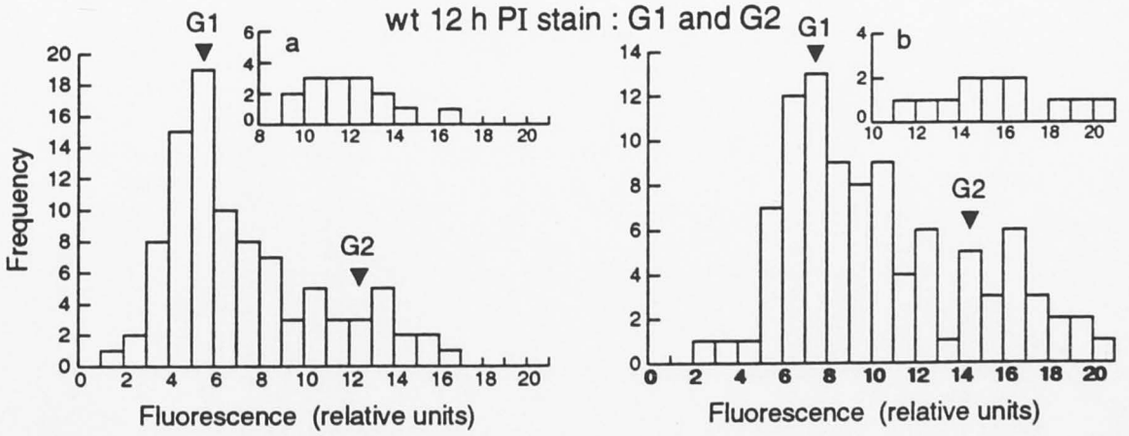


Fig. 4.4 Frequency of fluorescence values of nuclei in individual wild-type cells taken from synchronous culture at early G1 (0 h), late G1 (8 h) and G1, S and G2 period (12 h) using 5 µg/ml chromomycin A3.

Left panels represent values with background correction

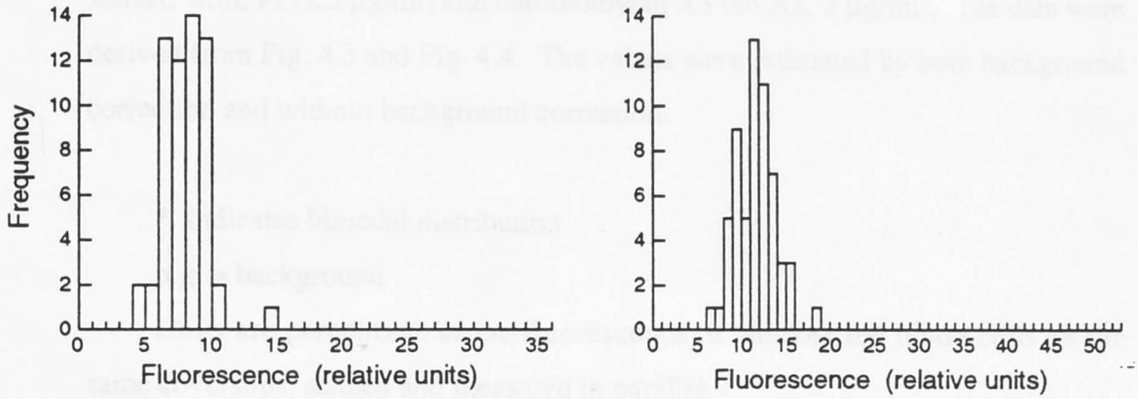
Right panels represent values without background correction

x-axis represents frequency; y-axis represents percentage of the fluorescence of chicken red blood cells stained and quantified in parallel.

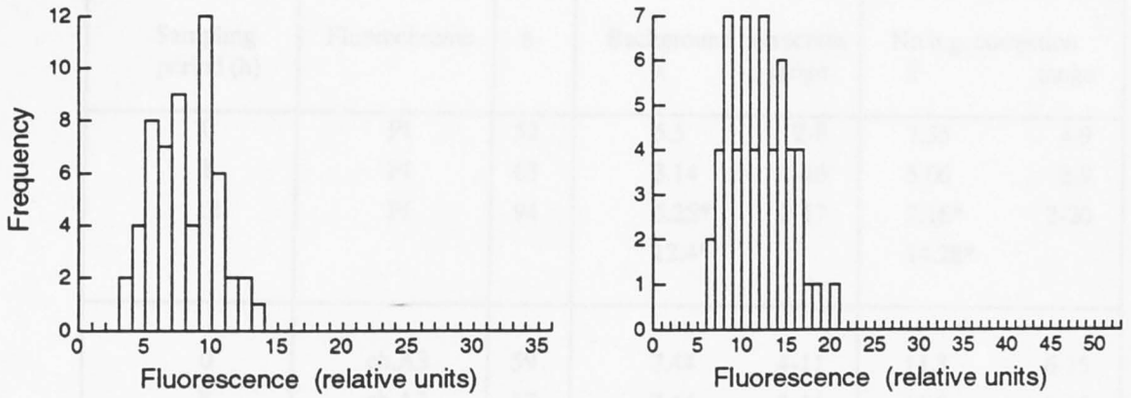
Background correction made

No background correction

wt 0 h ch.A3 stain : early G1



wt 8 h ch.A3 stain : late G1



wt 12 h ch.A3 stain : G1 and G2

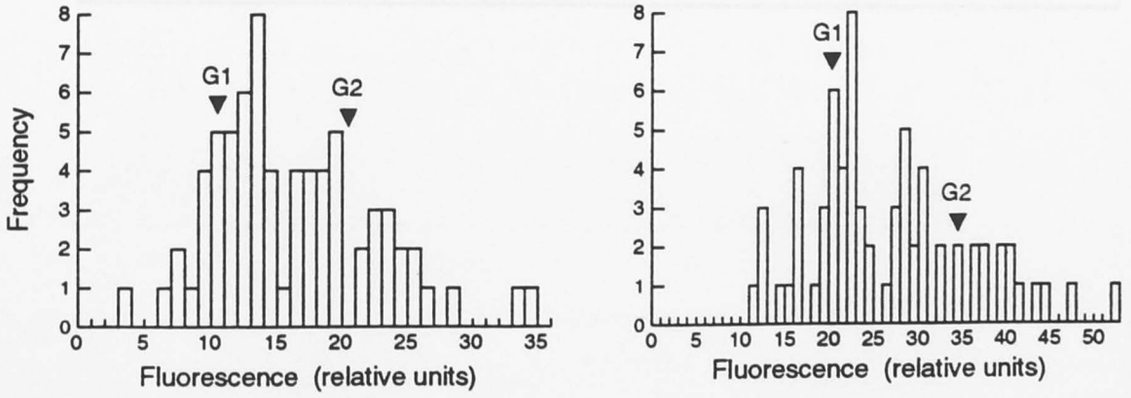


Table 4.5 Mean fluorescence values of nuclei in individual wild-type cells stained with; PI (2.5 μg/ml) and chromomycin A3 (ch.A3, 5 μg/ml). The data were derived from Fig. 4.3 and Fig. 4.4. The values were estimated by both background correction and without background correction.

* indicates bimodal distribution

b.g. = background

Units are percentage of the fluorescence of chicken red blood cells on the same coverslips, stained and measured in parallel.

Sampling period (h)	Fluorochrome	n	Background correction $\bar{x}$	range	No b.g. correction $\bar{x}$	range
0	PI	52	5.5	2-8	7.55	4-9
8	PI	63	3.14	1-10	5.06	2-9
12	PI	94	5.25*	1-17	7.16*	2-20
			12.4*		14.28*	
0	ch.A3	59	7.44	4-11	11.3	6-15
8	ch.A3	57	7.44	3-14	11.6	6-16
12	ch.A3	71	10.8*	3-28	19.35*	11-52
			20.6*		34.0*	

Fig. 4.5 Frequency of nuclear fluorescence values in mutants and in a positive control G2-arresting mutant stained with 2.5 µg/ml PI

Left panels represent values after background correction
Right panels represent values without background correction

a. and b. represent nuclear fluorescence values of mutant 92 cultured at 21°C and 33°C respectively

c. and d. represent nuclear fluorescence values of mutant 194 cultured at 21°C and 33°C respectively

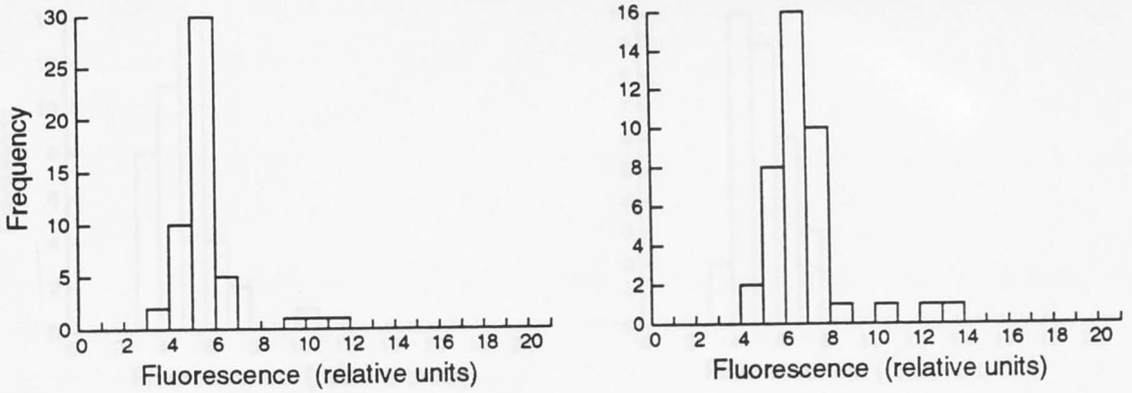
e. and f. represent nuclear fluorescence values of mutant 195 cultured at 21°C and 33°C respectively

g. and h. represent nuclear fluorescence values of a G2-arresting mutant (kindly provided by Dr J.D.I. Harper) cultured at permissive temperature (21°C) and non-permissive temperature (33°C). This mutant was used as a positive control of the method to confirm the detection of G1 (1C) DNA level of non-arrested cells and G2 (2C) level of arrested cells at non-permissive temperature.

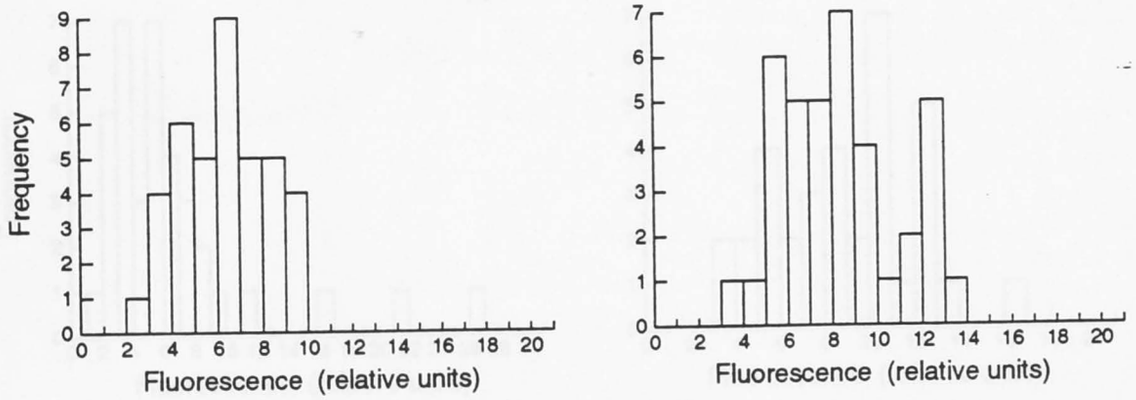
Fluorescence units are percentage of the fluorescence of chicken red blood cells on the same coverslip, stained and measured in parallel.

4.5 Background correction made No background correction

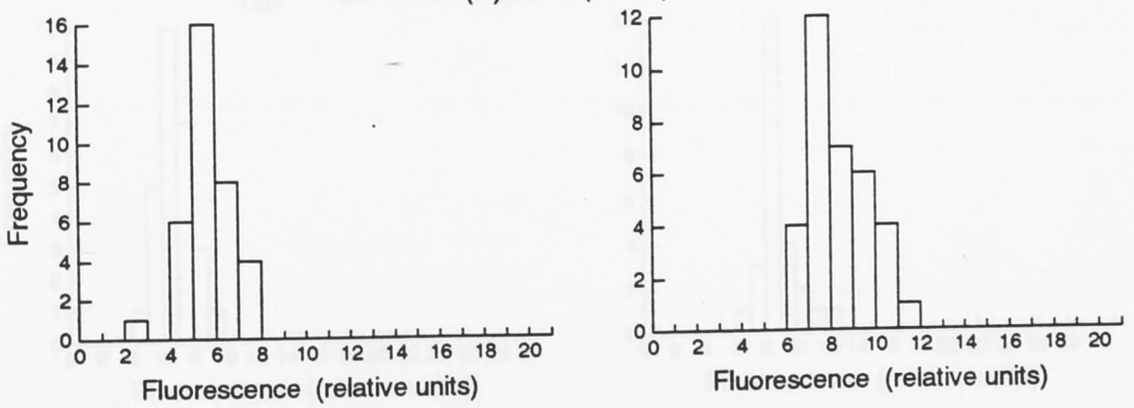
(a) 92 (21°C)



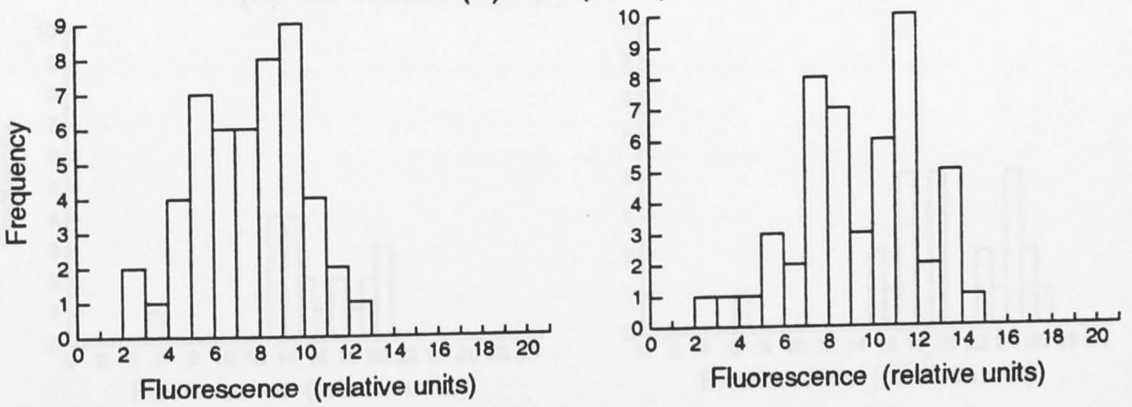
(b) 92 (33°C)



(c) 194 (21°C)

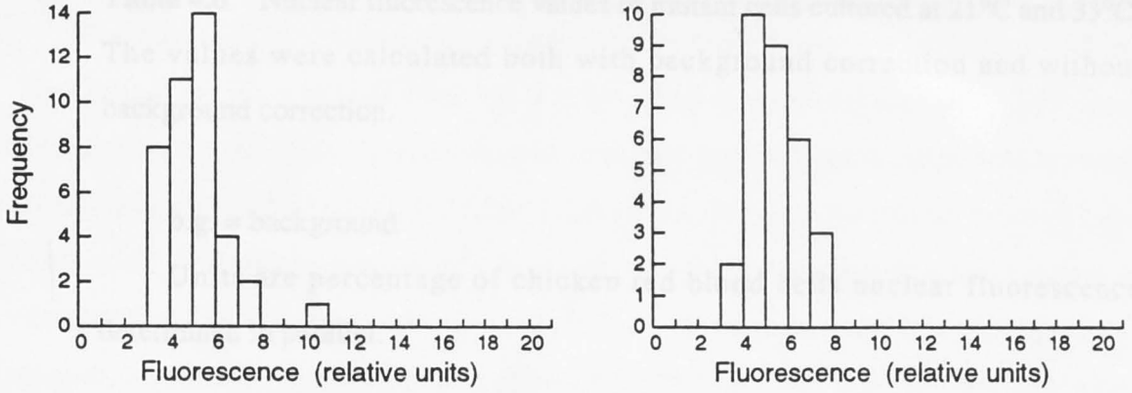


(d) 194 (33°C)

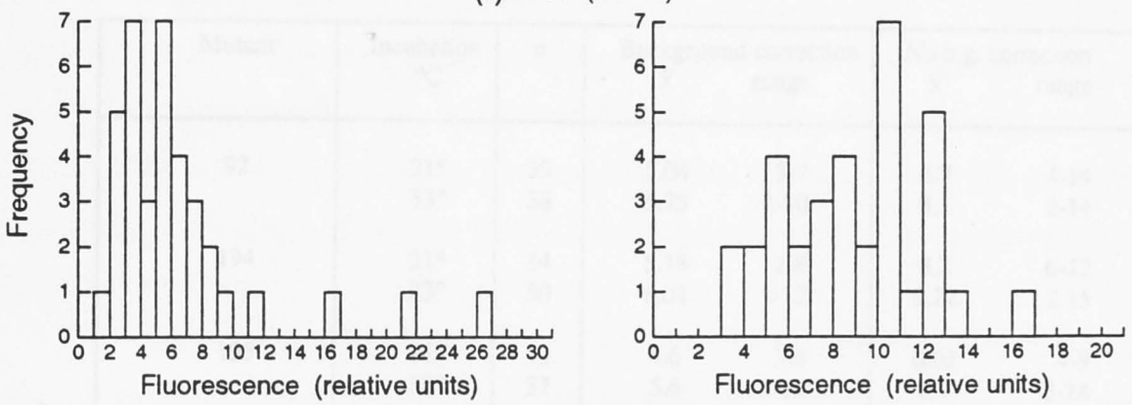


4.5 Background correction made — No background correction

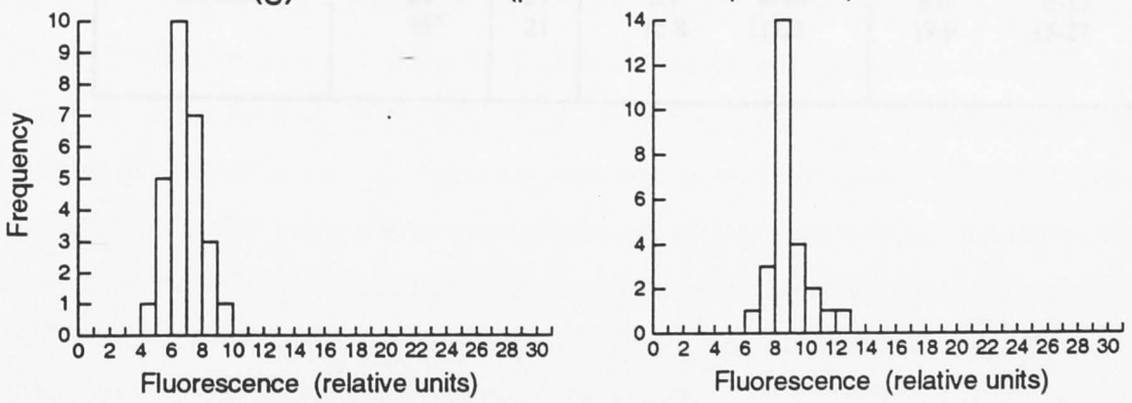
(e) 195 (21°C)



(f) 195 (33°C)



(g) G2 mutant (permissive temperature)



(h) G2 mutant (non-permissive temperature)

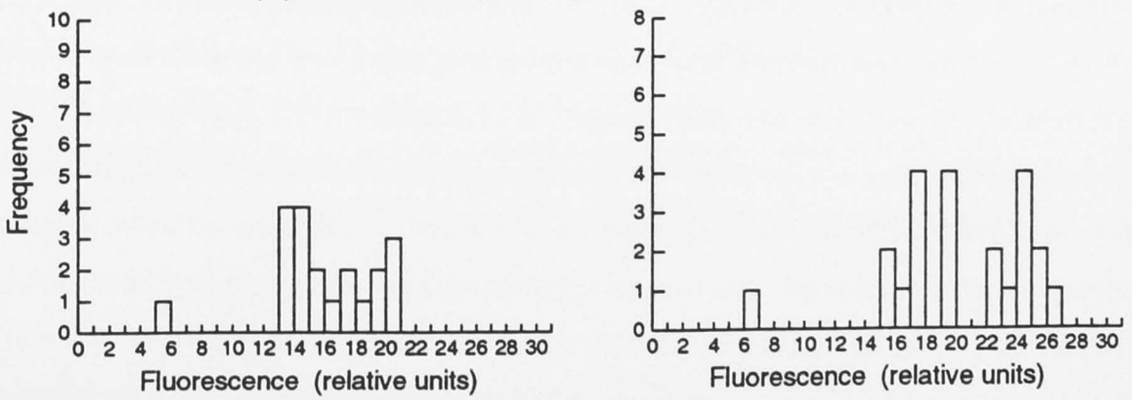


Table 4.6 Nuclear fluorescence values of mutant cells cultured at 21°C and 33°C. The values were calculated both with background correction and without background correction.

b.g. = background

Units are percentage of chicken red blood cells nuclear fluorescence determined in parallel.

Mutant	Incubation °C	n	Background correction $\bar{x}$	range	No b.g. correction $\bar{x}$	range
92	21°	39	5.04	3-7	6.7	4-14
	33°	38	5.75	2-10	8.1	3-14
194	21°	34	5.18	2-8	8.2	6-12
	33°	50	6.01	1-12	9.22	2.15
195	21°	40	4.6	3-8	6.01	4-9
	33°	37	5.6	1-10	8.7	3-14
G2 mutant	21°	27	6.7	4-10	8.8	6-13
	33°	21	15.8	11-21	19.9	15-27

Discussion

The nuclear DNA contents of mutant cells are crucial measurements by which the phase of cell cycle arrest is determined. The chemical methods for estimating DNA gave information on the total DNA content in each cell and did not discriminate between nuclear and non-nuclear DNA. In addition these methods were also subject to interference from other cellular constituents. Most organisms have a significant amount of extranuclear DNA, located for example, in the mitochondria and chloroplast. In *C. reinhardtii* quantitative preparation of chloroplast DNA indicates that it accounts for about 14% of the total DNA in haploid vegetative cells, that is about 1.72×10^{-8} µg/cell (Gillham, 1978). Mitochondria and the nuclear rDNA satelite (r) represents less than 1-2% of the total (Howell, 1972 ; Marco and Rochaix, 1980). The proportion of extra nuclear DNA will increase in enlarging division arrested cells and so will the amount of potentially interfering compounds. Thus a more specific method to detect only nuclear DNA was developed.

The method of quantifying nuclear DNA by microspectrophotometry made it possible to measure DNA in individual nuclei without any complex prior manipulation of the cells. There have been previous reports using microspectrophotometry to estimate cellular DNA content by measuring the fluorescence of individual nuclei isolated from the remainder of the field by the use of an appropriate combination of stage and measuring pinholes of a photomicroscope. These methods attempted to estimate DNA from a single measurement of the whole nucleus and assume that all light was focussed equally on the detector. For example the measurement of nuclear DNA of *C. ehrenbergii* using epifluorescence microspectrophotometry after DAPI staining (Hamada, et al., 1985 and 1987), and *C. reinhardtii* nuclear DNA by staining with DAPI and mithramycin (Coleman, 1981). However these methods would be interfered with cytoplasmic materials above the nucleus and would give greater error with large arrested mutant cells.

In conclusion, DNA estimation by fluorescence measurement can resolve the problems encountered with chemical assays methods which were biased by the amount of material taken for assay due to interference by some unknown compounds and were also influenced by nucleic acid from cytoplasmic organelles. By using the technique of fluorochrome staining, each nucleus can be measured directly and only small culture samples are required since the method is highly sensitive.

SECTION V TERMINAL PHENOTYPES OF ts-cAMP REQUIRING CDC MUTANTS

In this study, the terminal phenotypes of arresting cells were observed using specific antibodies as probes to investigate the cell cytoskeleton. The objective was to determine whether the structure of the cytoskeleton corroborated the evidence from nuclear DNA content that cells were arrested in G1 phase and to attempt to diagnose where in G1 phase the cells might be arrested. The microtubular cytoskeleton of *Chlamydomonas* have been well described at both the structural (Johnson and Porter, 1968; Cavalier-Smith, 1974; Coss, 1974) and immunofluorescence level (Doonan and Grief, 1987; Gaffal, 1988; Gaffal and Gammal, 1990). As in most eukaryotes, microtubules in *Chlamydomonas* are implicated in the organization of cytoplasmic architecture throughout the cell cycle and are particularly involved in division.

The antibodies used here were; anti-β-tubulin, anti-centrin, anti-acetylated tubulin, anti-basal body associated-microtubule organizing center (MPM-13) and antibody against non-histone phosphoprotein (MPM-2), which has shown a high level of phosphorylated protein in cells entering metaphase. These antibodies are explained in more detail in the following paragraphs with respect to their target proteins and their location during progress through the *Chlamydomonas* cell division cycle.

The tubulin-containing structures of intact cells of *Chlamydomonas* were studied using monoclonal antitubulin antibodies; anti-α- and anti-β-tubulin, by indirect immunofluorescence (Doonan and Grief, 1987). During interphase the cytoskeletal elements are found not only in flagella but also form a cortical array extending from flagellar roots (Ringo, 1967). Using antitubulin antibodies, flagellar root microtubules showed uniform staining along their lengths during interphase. The number of cytoplasmic microtubules per cell depends on the stage in the cell cycle. Newly divided cells which are in the process of flagellar growth have the smallest numbers of cytoplasmic rootlet microtubules (8-10) not all of which extend fully to the posterior end of the cell. Most fully flagellated cells have between 10-18 rootlet microtubules. Prior to mitosis, the flagella are either shed (Johnson and Porter, 1968) or they regress (Cavalier-Smith, 1974) and cells become round. At this stage the cell posseses the largest numbers of microtubules. In preprophase the basal body flagellar root complex separates and the two elements migrate to opposite poles of the nucleus. The cytoplasmic microtubules

continue to converge upon these elements but progressively depolymerise leaving only a few microtubules extending from each basal body which form the microtubular metaphase band (Gaffal and Gammal, 1990). At this stage the nucleus is elongated, more densely stained with DAPI, and it lies close to the cell surface. During early prophase spindle fibers spread from the spindle poles through fenestrae in the nuclear membrane and form a mitotic spindle. During metaphase, spindle fibers from the poles extend toward the nuclear midzone and chromosomes are organized on the metaphase plate. During anaphase, chromosomes separate and the midzone microtubules of the intranuclear spindle begin to disassemble, but outside the nucleus the metaphase band microtubules, derived from the persisting flagellar roots (Gaffal and Gammal, 1990) remain. At the transition between telophase and early cytokinesis, the spindle completely disappears and cortical microtubules begin to reappear again focussed on the basal bodies but particularly abundant in the cleavage furrow where they form a phycoplast. During cytokinesis the cleavage furrow ingrows from the cell periphery through the phycoplast and progressing more rapidly from the anterior end of the cell. Microtubules arise from the vicinity of the nucleus probably at the basal body-pericentriolar microtubule organizing center (MTOC) complex, fan out around the cell periphery and also toward the cell midline. The daughter nuclei migrate back toward each other and lie opposingly across the cleavage furrow. The cells may already have attained commitment to further cell doubling if mother cell mass allows (Donnan and John, 1983) and in this case before the cells separate completely they may reenter mitosis for a second, third or fourth time. All of these divisions involve similar arrays of microtubules, and all occur within the original mother wall. When cells do not reenter division, they become ellipsoidal and regenerate flagella and daughter cells are released from the mother cell wall.

MTOCs function as sites of microtubule initiation and are involved in the reorganization of cytoplasmic and mitotic microtubule arrays (Brinkley, 1985). The location and numbers of MTOCs was determined by the monoclonal antibody, MPM-13 which has been raised to the MTOC of Hela cells (Rao et al., 1989). The MPM-13 has been shown by immunofluorescence to react with MTOCs and to recognize proteins of similar molecular weight from various animal cells (Rao et al., 1989). In *Chlamydomonas* cells, MPM-13 has been shown to react with the basal body root complex, a component of a major MTOC (Harper et al., 1990a). This indicates conservation of MTOC components between plant and animal cells. The involvement of MPM-13 reacting

material in cell division was observed in wild-type cells in synchronous culture of *Chlamydomonas*. During the cell cycle the MPM-13 antigenic material was found to remain associated with the basal body flagellar root complex. During interphase, MPM-13 reacting material was found located anterior to the nucleus in the region where basal bodies are located and from where the flagellar root microtubules radiate. At pre-prophase, the flagella have withdrawn, the single pair of basal bodies have not yet duplicated and a single MPM-13 staining region was observed in the basal body root complex region. At prophase when basal bodies of *Chlamydomonas* duplicate (Gaffal, 1988), the MPM-13 reacting material also duplicated and began to migrate with the basal body complex toward opposite side of the nucleus. At metaphase the basal bodies of *Chlamydomonas* remain attached to the plasmalemma and are also located at the pole of the mitotic spindle. At cytokinesis, each of the two new daughter nuclei has a focus of MPM-13 reacting material located immediately anterior to them in the same region where the basal bodies are located and from which the developing flagellar roots extend.

Centrin is another cytoskeletal protein which is associated with the flagellar apparatus or centrosome of eukaryotic cells including algae, protozoa, mammals and higher plants (Salisbury et al., 1984, 1986, 1987, 1988; Schulze et al., 1987) and it shows calcium-sensitive contractile or elastic behavior (Salisbury et al., 1983; 1984; 1988). In mammalian cells, centrin homologs are components of centrioles and the spindle poles and spindle matrix of dividing cells (Salisbury et al., 1986). In lower eukaryotes centrin is a component of contractile striated flagellar roots and distal fibres (McFadden et al., 1978; Salisbury et al., 1984, 1987; Schulze et al., 1987). Molecular cloning studies showed that centrin is a member of the calcium-binding protein superfamily which includes parvalbumin, calmodulin and troponin (Salisbury, 1984). In *Chlamydomonas* centrin is a component of the distal fiber which links the two adjacent basal bodies to one another (McFadden et al.,1987; Salisbury, 1988) and it also links the flagellar apparatus to the nucleus through a pair of descending fibers. These fibers extend into the cytoplasm and branch into 8-16 fimbria, which curve around the nucleus. During interphase there is intense staining of the two fibers between the nucleus and the basal bodies and more diffuse staining of the fimbria around the nucleus (Wright et al., 1985). At the interphase/preprophase boundary, flagella are shed or regress and nuclei move towards the flagellar apparatus (Coss, 1974; Triemer and Brown, 1974). This coincides with the contraction of the centrin-based fiber system to form a tight aggregate of material on the

anterior region of the nucleus. During prophase the centrin cytoskeleton divides into two foci at the position of basal bodies. At metaphase, the separated centrin foci have moved to opposite poles of the nucleus, and outline a crescent shape spindle. At anaphase the crescent-shaped centrin array undergoes a second transient contraction and separation. During telophase the centrin array of each daughter nucleus reextends. At cytokinesis the centrin based cytoskelton has returned to an interphase organization. This process may repeat itself to give rise to four or eight daughter cells.

Phosphorylated proteins were detected using the monoclonal antibody MPM-2 which was initially raised against mitotic Hela cells. It has been shown to be mitosis specific and to recognize a subset of nonhistone phosphoproteins in a number of structures in eukaryotic cells (Davis et al., 1983). Most structures that react with MPM-2 either contain tubulin or are involved in its organization. These structures include prophase nuclei, mitotic MTOCs, kinetochores, spindles, chromosomes, midbodies, phragmoplasts and a soluble cytoplasmic fraction which appears in higher eukaryotic cells after nuclear envelope breakdown (Vandre et al., 1984, 1986). MPM-2 does not recognize mitotic MTOCs in *Chlamydomonas* cells but it appeared to stain nuclear envelope-associated phophoproteins especially concentrated in mitotic cells (Harper et al., 1990b). The reorganization pattern of MPM-2 reactive materials during the cell division cycle in *Chlamydomonas* was observed by Harper et al.(1990b). No stain was detected during interphase. When cells entered prophase, weak staining was observed in the nuclear envelope region. During metaphase, MPM-2 stained the region around the nucleus with more intense staining on the anterior area. The nuclear staining reached a maximum intensity at the metaphase/anaphase boundary and staining still continued to surround the nuclear envelope throughout anaphase, but was most intense in the mid-anterior envelope region when the nucleus and spindle were elongating. By telophase, staining intensity dropped rapidly towards premitosis background levels. This temporal order of the MPM-2 staining pattern was the same whether a cell was dividing for the first, second or third time.

Acetylated tubulin was detected using the anti acetylated α-tubulin monoclonal antibody which was produced by the hybridoma 6-11B-1 (Piperino and Fuller, 1985). This antibody has been used to detect the distribution of acetylated α-tubulin in interphase cells of *Chlamydomonas* (LeDizet and Piperino, 1986). The acetylated α-tubulin is not only found in axonemes but is also present in basal bodies and in a subset of cytoplasmic

microtubules that radiate for a short distance from the basal bodies just beneath the plasma membrane. The role of α-acetylation in microtubules is unknown, however it was suggested to be part of a mechanism for stabilizing microtubules (LeDizet and Piperino, 1986). Since acetylated α-tubulin was also present in the basal bodies, therefore antiacetylated α-tubulin antibody can be used as well as MPM-13 antibody to detect the changes of basal bodies during the cell division cycle in *Chlamydomonas*.

Terminal phenotypes of arrested mutant cells

Mutants 194, 195 and 92 were stained with 5 different antibodies; MPM-2, anti-β-tubulin, MPM-13, anti-acetylated-tubulin and anti-centrin. Arrested cells of mutants 194, 195 and 92 all showed no staining with MPM-2 antibody. This indicated that they were not arrested in mitosis and it correlated with the G1 arrest indicated by DNA content. The arrest point was more precisely defined as probably in very late G1 by staining of other cytoskeletal components.

Staining of tubulin indicated that the microtubular cytoskeleton of the three mutants when grown at 21°C was normal. The cortical microtubules and flagella stained uniformly along their length with anti-β-tubulin antibody. The cortical microtubules converged on the flagellar apparatus (Fig. 5.1a). At 33°C, when division was arrested, cells lost their flagella, increased in size and become rounded and by 24 h were more than fifteen times the volume of unarrested cells. Cortical microtubules increased in number and continued to radiate from the flagellar roots. This microtubule organization of regressed flagella but fully polymerised root and cortical microtubules was typical of cells in late G1 after commitment to division (Figs. 5.1b, c and d).

Basal bodies of these cells were observed by staining with MPM-13 antibody and anti-acetylated-tubulin antibody. In cells at 21°C only a single area of MPM-13 staining was observed at the anterior end just beneath flagella indicating a single pair of basal bodies (Fig. 5.2a). In the same cells the anti-acetylated-tubulin stained flagella, basal bodies and a subset of root microtubules immediately adjacent to the basal bodies (Fig. 5.3a). In arrested cells antiacetylated-tubulin antibody staining showed only one pair of the basal bodies in each cell (Figs. 5.3b, c and d) which correlated with the evidence of MPM-13 staining (Figs. 5.2b, c and d). No flagella appeared in any of the arrested cells using anti-acetylated-tubulin antibody (Figs. 5.3b, c and d). Therefore the basal bodies of the arrested cells of all three mutants have not yet duplicated indicating that mitosis is not

imminent although the flagella have been withdrawn, which shows that commitment to division has been attained.

Using the anti-centrin antibody, cells at 21°C showed typical interphase staining of centrin, i.e., a pair of intensely stained fibrelike strands (the connector) was observed between the nucleus and an additional more diffuse staining extending round the nucleus from the anterior (Fig. 5.4a). At 33°C, the connector fibers remained detectable which indicated that centrin had not begun to contract as it would at G2. The basketlike array around the nucleus was diffuse. This pattern is characteristic of cells prior to G2 phase (Figs. 5.4b, c and d).

The use of five different antibodies to observe the arrested phenotypes of these three mutants indicated that all three were arrested at the same or similar stage of the cell cycle in the absence of exogenous cAMP/db-cAMP at non permissive temperature. The arrested cells had grown to a large size and increased in cortical microtubules, which is characteristic of late G1. There was also evidence that they had become committed to divide because they had lost or withdrawn their flagella. However there was no evidence that they had begun even the earliest detectable preparation for mitosis. The duplication of basal bodies is an indication of preparation for mitosis and staining with MPM-13 and anti- acetylated tubulin antibodies indicated that mutants were arrested before the duplication of basal bodies occurred. The contraction of the centrin connector is another early indicator of cell preparation for mitosis. The centrin connectors of arrested cells were still detectable which indicated contraction had not occurred.

This diagnosis is strongly supported by the examination of DNA content which indicated that the mutants under nonpermissive condition were arrested with G1 DNA content and therefore preparation for mitosis would have been inappropriate. Taken together these observations indicated that the mutant cells were arrested in late G1 phase of the cell cycle probably having completed the START division, or commitment to division but had not progressed to S phase (section IV) in the absence of normal levels of cAMP (section VIII) and because of the dependency of nuclear division on completion of S phase (Nurse, 1990), the cells had not made any detectable preparation for mitosis. Dependency of nuclear division on completion of S phase is widely observed in eukaryotes including budding yeast, fission yeast and *Chlamydomonas* (Harper and John, 1986).

Fig. 5.1 Cells stained with anti β-tubulin

a. anti β-tubulin staining of a newly divided cell which was cultured at 21°C. This young cell is clearly ellipsoid and the antibody stained cortical microtubules and flagella.

b, c and d show β-tubulin staining of mutant cells after arrest at 33°C for 24 h. The mutants show very similar terminal phenotypes at non permissive temperature in the absence of cAMP/db-cAMP. Cells were not able to divide but continued growth and became rounded. Microtubules were increased in number and converged upon the basal bodies at the anterior of the cell. Flagella have regressed. This is the typical organization of cells in late G1 that have passed commitment and are about to progress to S phase then mitosis.

b. anti β-tubulin staining of mutant 92 after arrest at 33°C for 24 h

c. anti β-tubulin staining of mutant 194 after arrest at 33°C for 24 h

d. anti β-tubulin staining of mutant 195 after arrest at 33°C for 24 h

Scale bar = 10 μm

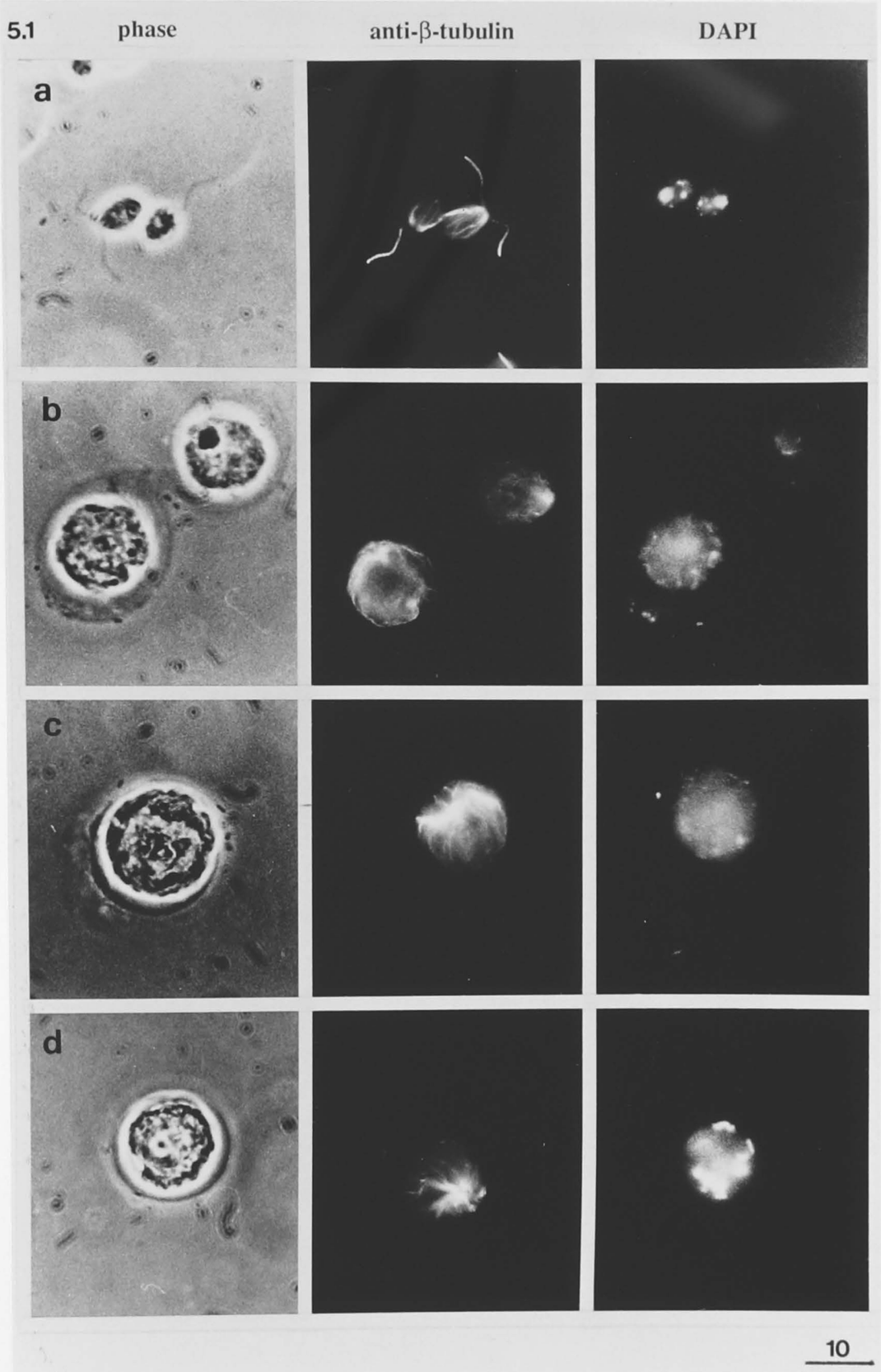

5.1
phase
anti-β-tubulin
DAPI
a
b
c
d
10

Fig. 5.2 MPM-13 stained cells

MPM-13 antibody reacts with basal body root complex (basal body pair) of *Chlamydomonas*. Positive staining was seen as a white spot located at the anterior of the cell immediately beneath the area where flagella emerge. The flagella are continuous with the basal bodies but only the basal body pair stains with MPM-13.

a. MPM-13 staining of cells cultured at 21°C. A single area of MPM-13 stain was seen at the anterior end of the cell.

b, c and d show MPM-13 staining of mutant cells after arrest at 33°C for 24 h in the absence of cAMP/db-cAMP. All three mutants were arrested with a single pair of basal bodies, seen as one bright dot.

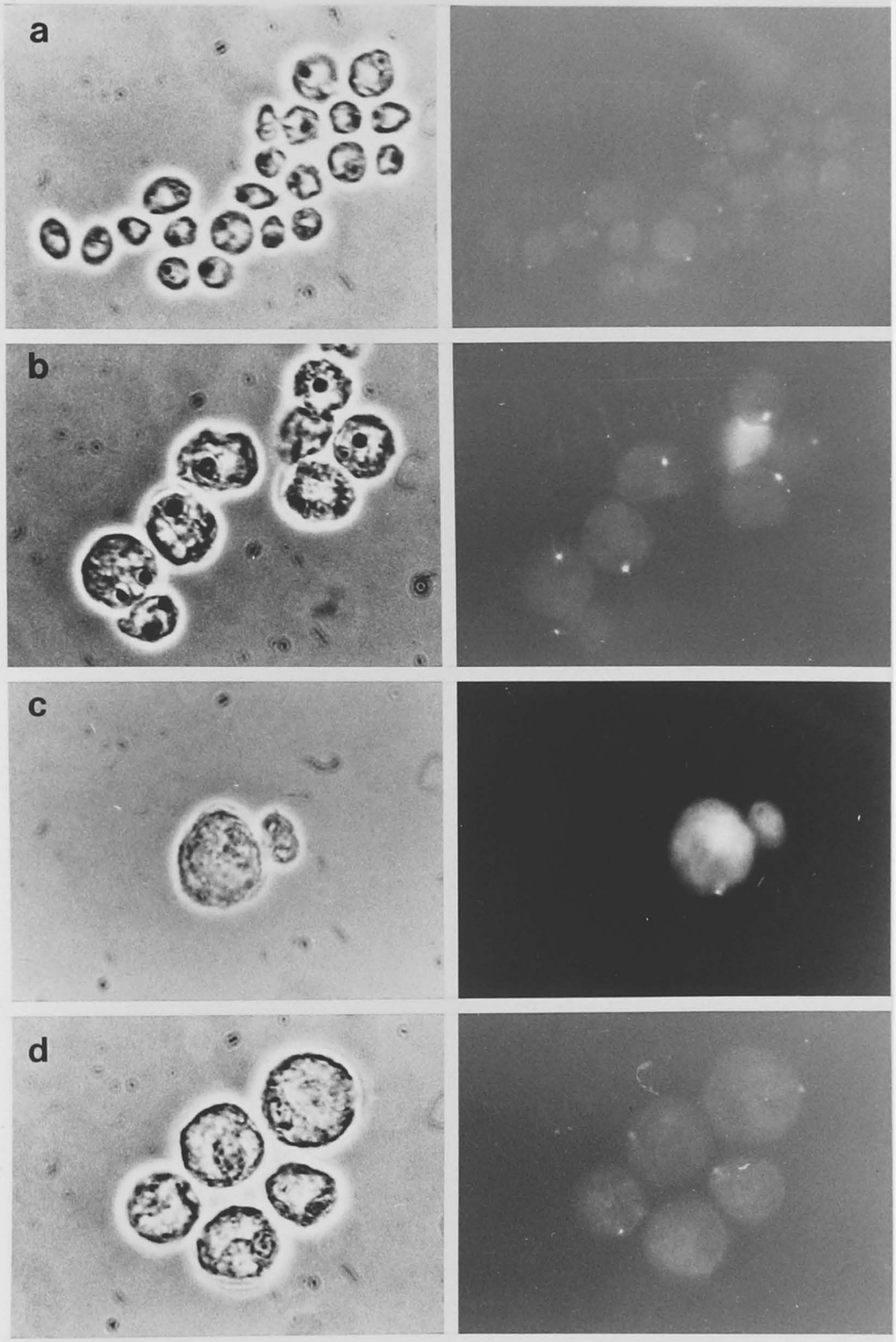
b. MPM-13 staining of mutant 92 after arrest at 33°C for 24 h

c. MPM-13 staining of mutant 194 after arrest at 33°C for 24 h

d. MPM-13 staining of mutant 195 after arrest at 33°C for 24 h

Scale bar = 10 μm

5.2 phase MPM-13



10

Fig. 5.3 Cells stained with anti-acetylated tubulin antibody

Anti-acetylated tubulin antibody detects flagella, basal bodies and a subset of rootlet microtubules adjacent to the basal bodies in the *Chlamydomonas* cell.

a. anti-acetylated tubulin staining of cells at 21°C. Typical interphase staining was seen, showing flagella (f), a single pair of basal bodies (bb) which were continuous with the flagella and the rootlet microtubules (r) which radiated from the basal bodies

b, c and d show anti-acetylated tubulin staining of mutant cells. Cells were arrested at 33°C for 24 h in the absence of cAMP/db-cAMP. Anti-acetylated tubulin antibody showed that all the mutant cells were arrested at a similar stage of the cell cycle. Mutants were arrested with a single pair of basal bodies, the flagella had regressed and the cells had grown large and become round but no detectable preparation of mitosis such as duplication of basal body pairs had occurred.

b. anti-acetylated tubulin staining of mutant 92 after arrest at 33°C for 24 h

c. anti-acetylated tubulin staining of mutant 194 after arrest at 33°C for 24 h

d. anti-acetylated tubulin staining of mutant 195 after arrest at 33°C for 24 h

Scale bar = 10 μm

5.3

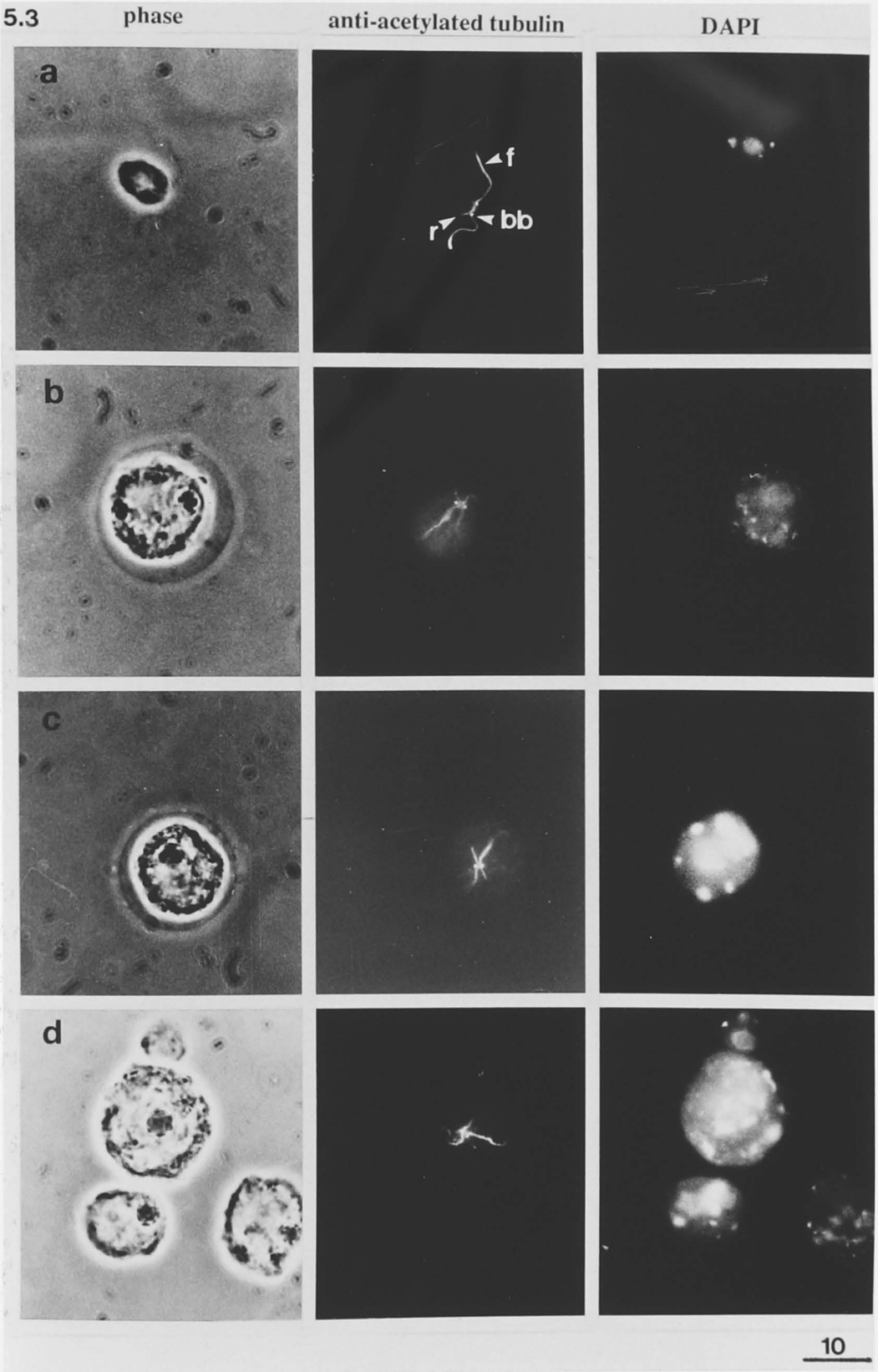


Fig. 5.4 Anti-centrin stained cells

Anti-centrin antibody detects a component of the distal fiber, which links the two adjacent basal bodies of the pair and it also links the flagellar apparatus to the nucleus.

a. anti-centrin staining of cells cultured at 21°C. Anti centrin antibody showed intense staining of the two connector fibers (c) between the nucleus and basal bodies and faint staining of the fimbria (f) around the nucleus.

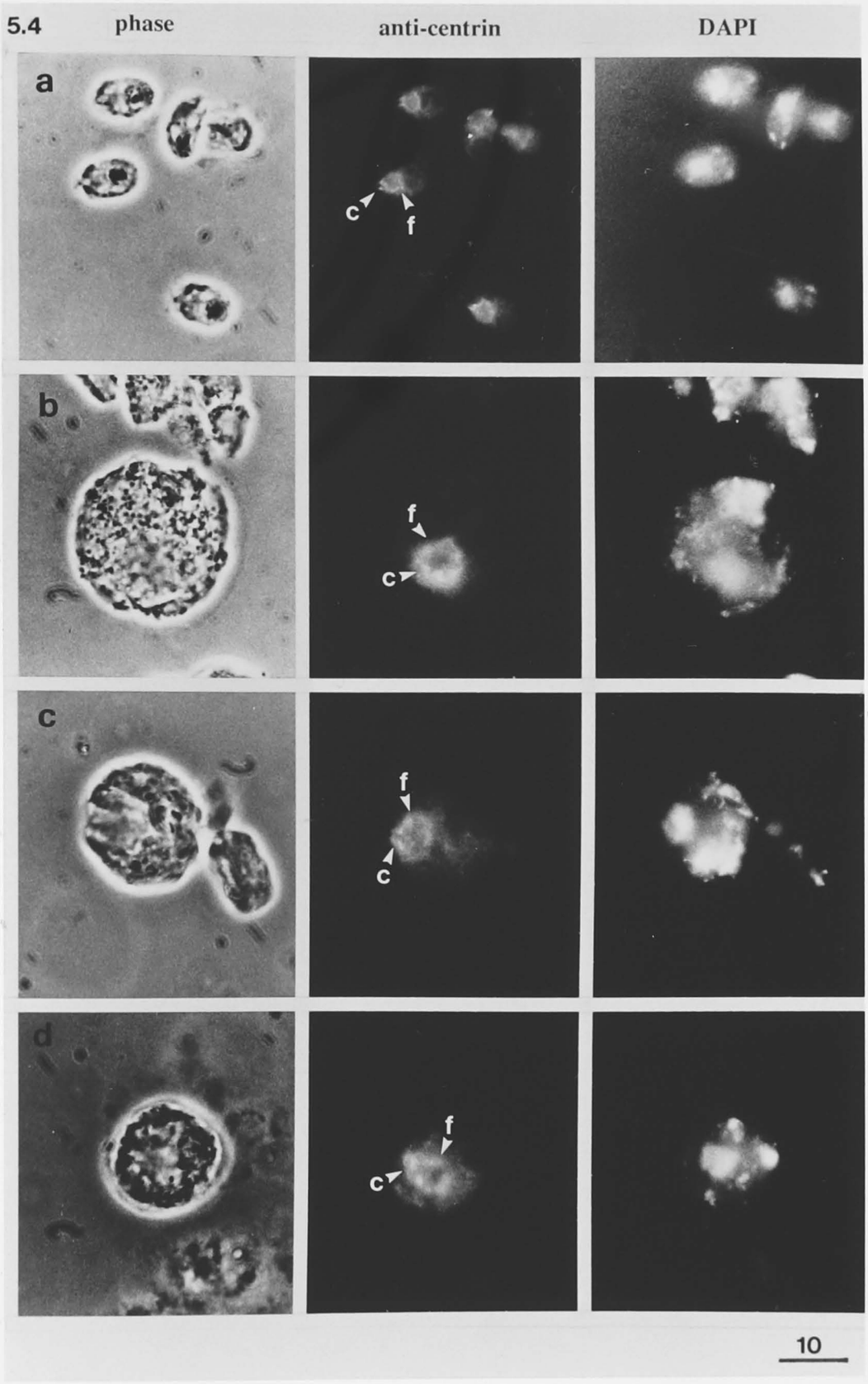
b, c and d show anti-centrin staining of mutant cells after arrest at 33°C for 24 h in the absence of cAMP/db-cAMP. Mutant cells were arrested with the same pattern of centrin organization. At 33°C the connector between the nucleus and basal bodies has not contracted and the basket array (fimbria) around the nucleus is still present staining faintly, indicating that mitosis is not imminent.

b. anti-centrin staining of mutant 92 after arrest at 33°C for 24 h

c. anti-centrin staining of mutant 194 after arrest at 33°C for 24 h

d. anti-centrin staining of mutant 195 after arrest at 33°C for 24 h

Scale bar = 10 μm


5.4
phase
anti-centrin
DAPI
a
b
c
d
c
f
10

SECTION VI GENETIC ANALYSIS OF ts cAMP-REQUIRING CDC MUTANTS

To investigate the genes that in mutated form were responsible for the phenotypes of the mutants, each was repeatedly back crossed to the wild type strain. The objectives of back crossing were to determine how many genes contributed to the observed phenotype and also to remove any extraneous mutations that may have been induced by the EMS treatment and might otherwise modify some aspects of the behaviour of the mutant cells. At each back cross a complete set of entirely normal genes was introduced by the wild-type mating partner. Independent Mendelian segregation then allowed possible unwanted mutations to be discarded by chance into offspring other than those selected as having the mutation of interest. Repeated back crossing should result in mutants with an entirely wild-type genetic background. Thus effects entirely due to the genes selected for study can be observed without possible distortion due to the presence of other altered genes.

For analysis of the progeny from back crosses, meiotic products were tested for their ability to grow on 3 mM caffeine plates at 21°C and to block in division at 33°C while growth in cell size continued on plates containing no inhibitor. Progeny that were temperature sensitive on plates were further investigated in liquid medium with and without supplementation by cAMP or db-cAMP at 33°C. The results of back crosses indicated that in each original isolate four classes of genes were contributing to the phenotype. In each isolated mutant the same four types of gene were present, although in some cases genetic analysis revealed that mutation in identical genes could not be involved. For example the genes conferring cdc arrest and caffeine resistance showed different linkage in different mutants.

The evidence that although the same four types of gene were present in each of the mutants they were not repeated isolation of the same mutagenic event came initially from their clearly distinguishable properties when first isolated. One difference was in the extent of rescue by cAMP at 33°C (described in section III). All cells of the original isolate of mutant 92 entered division in response to cAMP but some cells of the original isolates of mutant 194 and 195 remained blocked. They also differed in mating performance on first back cross. Germinating zygospores of 92 and 194 routinely gave wholly viable sets of progeny but 195 gave predominantly sets of offspring in which most were non-viable. Subsequent genetic analysis showed that the linkage between the gene giving the cdc phenotype *(cdc C)* on agar at 33°C and the gene giving caffeine resistant

(caf R) was very different in each mutant. In mutant 92 these two genes are so closely linked that no recombination was detected in offspring from 74 zygospores, while in mutant 195 they are located 7.3 m.u. (mapping unit) from each other. In mutant 194 the distance between the two genes is 25.9 m.u. which indicates greater separation. Presumably either different *cdc C* or *caf R* genes were present in the three mutant isolates that could be analysed genetically.

Partial phenotypes as well as the full parental phenotype were observed among the progeny from back crosses presumably because of the inheritance of only some of the genes that contributed to the full phenotype of the original isolate. Since the same phenotypes were observed from back crosses of all three isolates the following descriptions can serve as a general introduction.

Three phenotypes that affected division arrest and rescue in liquid medium at 33°C were observed.

Type I: cells behaved like the mutant parent. In the absence of cAMP or db-cAMP in liquid at 33°C they were blocked in division but continued growing in size and they were restored to division by supplementation with cAMP or db-cAMP while 5'-AMP was not effective. It should be noted that the original isolates of mutants 194 and 195 could not be completely rescued by cAMP, but after back crossing all could be rescued by cAMP. The pattern of cell number increase under conditions of arrest and rescue of cells with this phenotype are shown in Fig. 6.1 for mutants 92, 194, 195 after repeated back cross.

Type II: cells were not affected in division by the presence or absence of cAMP or db-cAMP and were blocked in division in liquid at 33°C but continued growing in size. None of the chemicals supplied could promote resumption of division. The pattern of cell number increase of this cell type is shown in Fig. 6.2.

Type III: cells divided normally at 33°C in liquid although division was arrested on agar and in liquid culture all of the supplemented chemicals promoted an increase in final cell number close to wild-type density. The increase was commonly 1.5 times the unsupplemented number and was largely due to raising of the stationary cell density closer to wild-type level rather than faster growth. The pattern of cell number increase of this cell type is shown in Fig. 6.3.

Fig. 6.1 Pattern of cell number increase under conditions of arrest or rescue of cells by cAMP or db-cAMP;
Cells were grown in TAPYPP medium at 21°C until they reached exponential phase then the cells were diluted into fresh TAPYPP medium and incubated at 21°C and 33°C, and TAPYPP medium supplemented with cAMP or db-cAMP and incubated at 33°C.

Either cAMP or db-cAMP was tested at the concentration of 1 mM and 5 mM. The most effective concentrations were indicated in the figure legend. AMP and butyrate were added at the same final concentration as cAMP and db-cAMP.

a. arrest phenotype I of mutant 92 (cAMP 5 mM, db-cAMP 5mM)

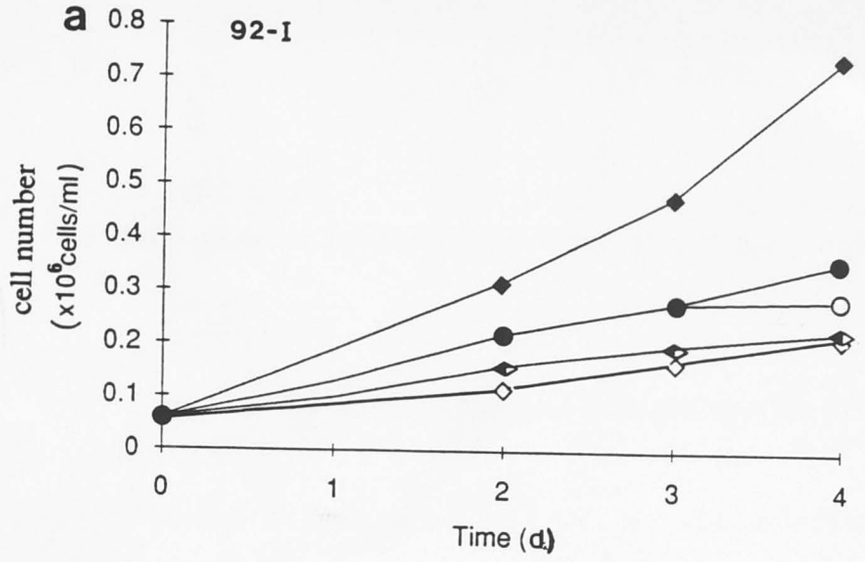
b. arrest phenotype I of mutant 194 (cAMP 1 mM, db-cAMP 5 mM)

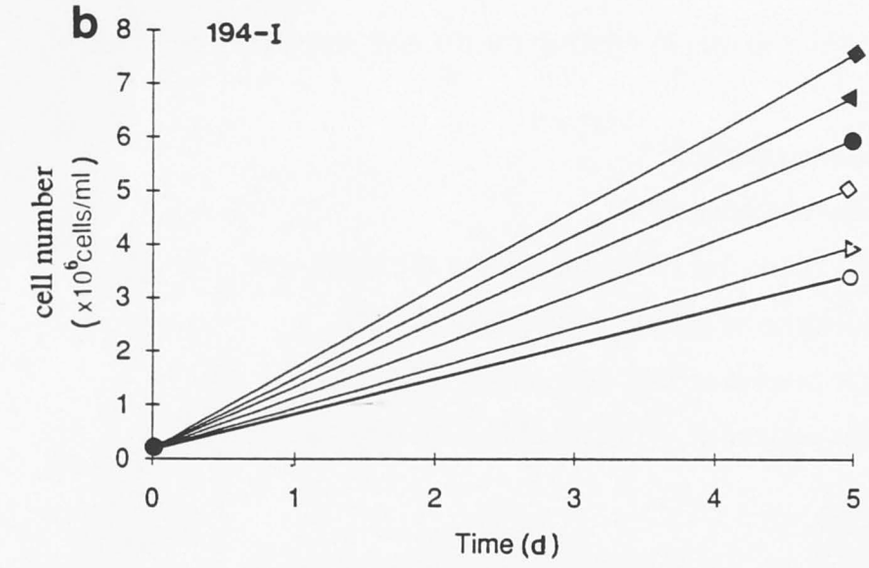
c. arrest phenotype I of mutant 195 (cAMP 1 mM, db-cAMP 5 mM)

Symbols:
- ● cells cultured at 21°C.
- ○ cells cultured at 33°C.
- ◀ cells cultured at 33°C with addition of cAMP.
- ▷ cells cultured at 33°C with addition of AMP.
- ◆ cells cultured at 33°C with addition of db-cAMP.
- ◇ cells cultured at 33°C with addition of butyrate.

d = days

6.1


a
92-I
cell number
(x10^6cells/ml)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
Time (d)


b
194-I
cell number
(x10^6cells/ml)
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
Time (d)

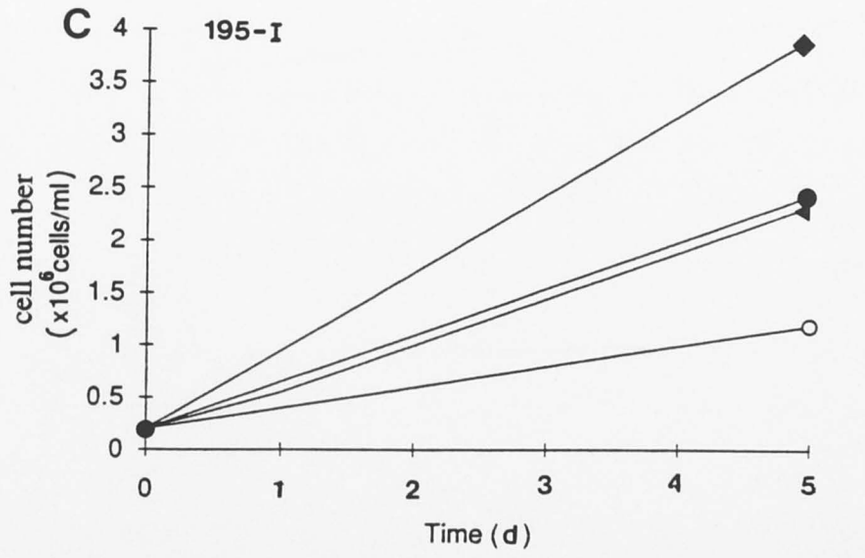

C
195-I
cell number
(x10^6cells/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
Time (d)

Fig. 6.2 Pattern of cell number under conditions of arrest and rescue by cAMP or db-cAMP;

Cells were cultured in the same condition as described in Fig. 6.1.

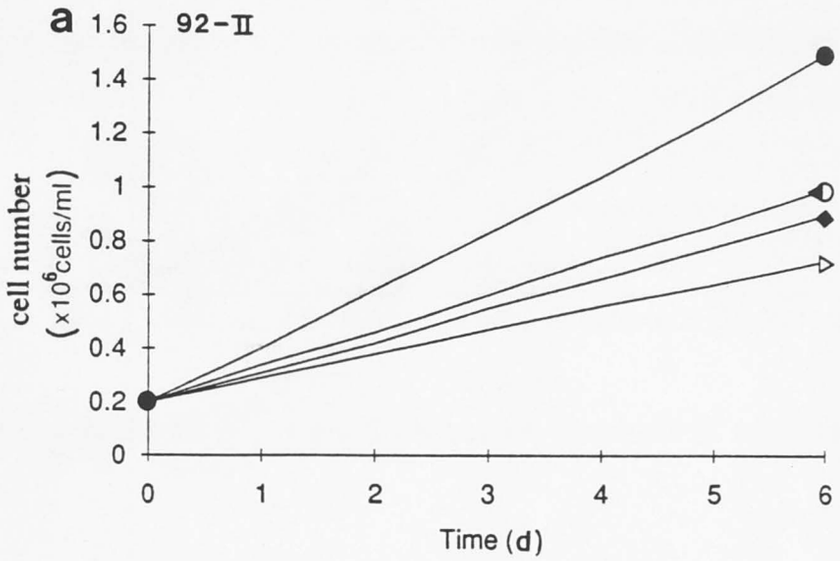
a. arrest phenotype II of mutant 92 (cAMP 1 and 5 mM, db-cAMP 1 and 5 mM)

b. arrest phenotype II of mutant 195 (cAMP 1 and 5 mM, db-cAMP 1 and 5 mM)

Symbols:
- ● cells cultured at 21°C.
- ○ cells cultured at 33°C.
- ◀ cells cultured at 33°C with addition of cAMP.
- ▷ cells cultured at 33°C with addition of AMP.
- ◆ cells cultured at 33°C with addition of db-cAMP.
- ◇ cells cultured at 33°C with addition of butyrate.

d = days

6.2


a
92-II
cell number
(x10^6cells/ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
Time (d)

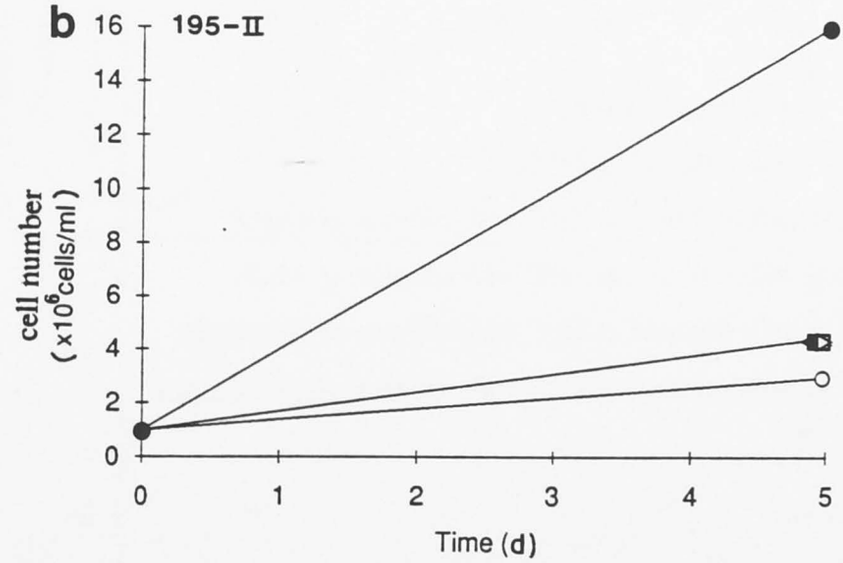

b
195-II
cell number
(x10^6cells/ml)
16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
Time (d)

Fig. 6.3 Pattern of cell number under the condition of arrest and rescue by cAMP or db-cAMP;

Cells were cultured as described in Fig. 6.1.

a. arrest phenotype III of mutant 92 (cAMP 1 and 5 mM, db-cAMP 1 and 5 mM)

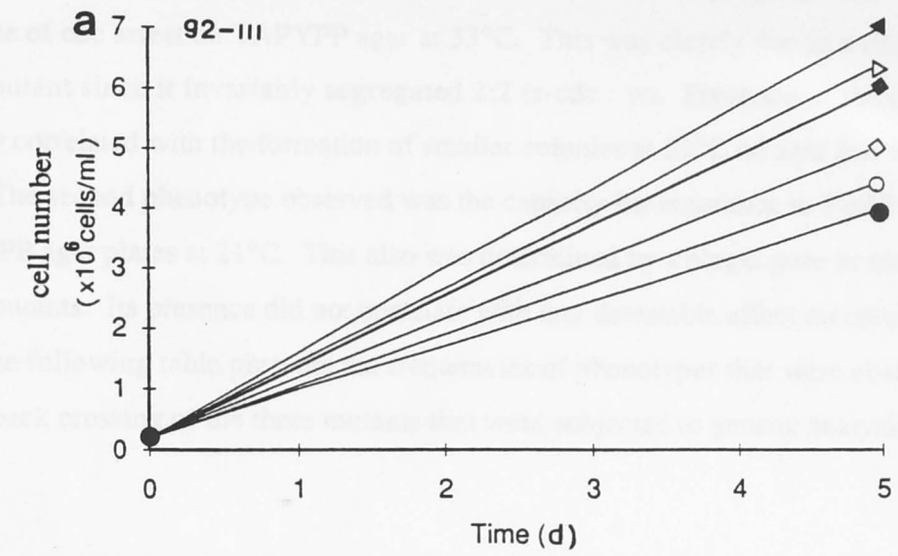
b. arrest phenotype III of mutant 194 (cAMP 1 and 5 mM, db-cAMP 1 and 5 mM)

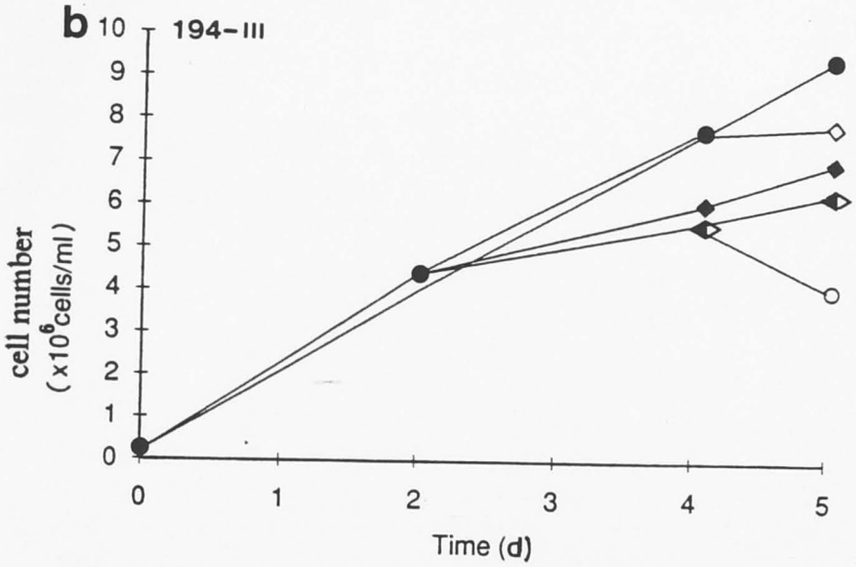
c. arrest phenotype III of mutant 195 (cAMP 1 and 5 mM, db-cAMP 1 and 5 mM)

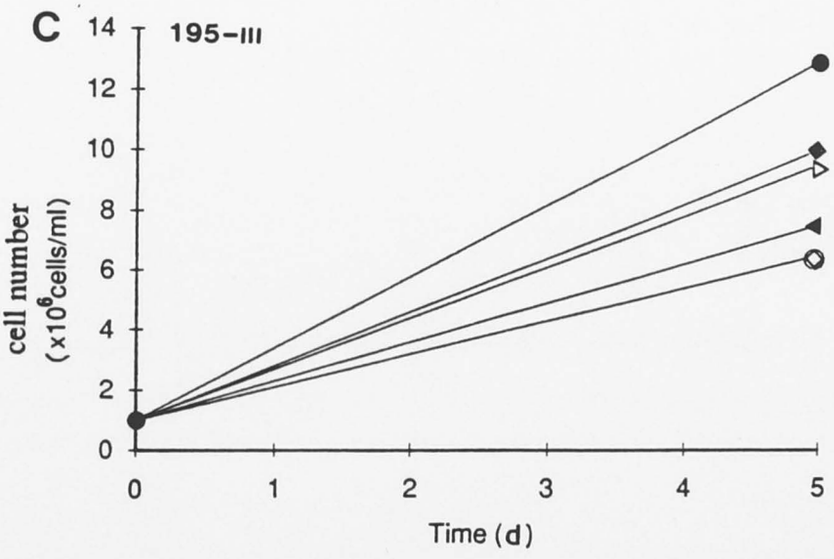
Symbols:
- ● cells cultured at 21°C.
- ○ cells cultured at 33°C.
- ◀ cells cultured at 33°C with addition of cAMP.
- ▷ cells cultured at 33°C with addition of AMP.
- ◆ cells cultured at 33°C with addition of db-cAMP.
- ◇ cells cultured at 33°C with addition of butyrate.

d = days

6.3


a
92-III
cell number
(x10^6cells/ml)
Time (d)


b
194-III
cell number
(x10^6cells/ml)
Time (d)


C
195-III
cell number
(x10^6cells/ml)
Time (d)

Two additional phenotypes were scored for each of the progeny. One was the occurrence of cdc arrest on TAPYPP agar at 33°C. This was clearly due to a single gene in each mutant since it invariably segregated 2:2 ts-cdc : wt. Presence of the cdc gene invariably correlated with the formation of smaller colonies at 21°C on agar due to slower growth. The second phenotype observed was the capacity for resistance to 3 mM caffeine on TAPYPP agar plates at 21°C. This also was determined by a single gene in each of the isolated mutants. Its presence did not correlate with any detectable effect on colony size.

The following table presents the frequencies of phenotypes that were observed on repeated back crossing of the three mutants that were subjected to genetic analysis.

Table 6.1 Summary of the results of tetrad analysis and descriptions of terminal phenotypes on testing cells at 33°C on agar plates and liquid medium, and their ability to grow on 3 mM caffeine plates at 21°C.

Mutants 92, 194 and 195 were backcrossing repeatedly with wild-type. Temperature-sensitive colonies of the progeny were further tested in liquid medium at 33°C to observed the response of the cells to cAMP/db-cAMP supplementation. The terminal phenotypes were scored as falling into three main types:

a. type I arrest: cells grew normally at 21°C but blocked in division at 33°C in liquid. Supplementation with cAMP or db-cAMP restored cell division.

b. type II arrest: cells grew slowly at 21°C but blocked in division at 33°C in liquid. Supplementation with cAMP/db-cAMP did not restore division.

c. type III arrest: cells grow and divide normally at 21°C and 33°C in liquid. Supplementation cAMP/db-cAMP increased stationary phase cell number.

Columns labelled progeny a, b, c, d are arbitrarily named offspring from single tetrads. The column " number of zygospores " indicates the number of zygospores germinated from which progeny were test on agar plates. Progeny from a smaller number of these zygospores were simultaneously tested in liquid medium as indicated by the heading " Total zygospores tested in liquid ". Brackets indicate the same phenotypes on plates with respected to *cdc ts* (ts) and *caf R*. The column "F generation" is both number of previous generations of back cross and in brackets indicate the arrest phenotype of mutant parent in the current back cross.

Example; the first compartment in the table indicates that 17 zygospores of 92 F1 were analysed, all showed a 2:2 ratio of ts-caff : wt on agar; further tests in liquid medium of sets progeny from five zygospores showed that I:II: wt:wt was observed in two sets of progeny; I:III:wt:wt was observed in two other sets and III:III:wt:wt was observed in one set.

6.1

Mutant	F Generation	Number of Zygospores	Phenotypes of progeny.								Total Zygospores Tested in Liquid
			Progeny a		Progeny b		Progeny c		Progeny d		
			plate	liquid	plate	liquid	plate	liquid	plate	liquid	
92	F1	17 {	ts, caf	I	ts, caf	II	wt	-	wt	-	2
			ts, caf	I	ts, caf	III	wt	-	wt	-	2
			ts, caf	III	ts, caf	III	wt	-	wt	-	1
194	F1	{	ts, caf	III	ts, caf	III	wt	-	wt	-	1
		6 {	ts, caf	II	ts, caf	III	wt	-	wt	-	1
			ts, caf	I	ts, caf	III	wt	-	wt	-	1
		2	ts, caf	-	ts	-	wt, caf	-	wt	-	
		1	ts	-	ts	-	wt	-	wt	-	
195	F1	2	ts, caf	III	NG	-	NG	-	NG	-	1
		2	ts, caf	III	ts, caf	I	NG	-	NG	-	1
		2	ts, caf	III	ts	-	NG	-	NG	-	1
		2	ts	-	NG	-	wt	-	NG	-	
		1	ts, caf	-	ts	-	wt, caf	-	NG	-	
		1	NG	-	NG	-	wt, caf	-	wt, caf	-	
92	F2 (I)	16 {	ts, caf	I	ts, caf	III	wt	-	wt	-	2
			ts, caf	III	ts, caf	III	wt	-	wt	-	1
194	F2 (I)	1	ts, caf	I	ts, caf	III	wt	-	wt	-	1
		1	ts, caf	I	ts, caf	-	wt	-	wt	-	1
		1	ts, caf	III	ts, caf	-	wt	-	wt	-	1
		1	ts, caf	III	ts	III	wt	-	wt	-	1
	F2 (III)	2	ts, caf	III	ts, caf	III	wt	-	wt	-	1
		2	ts, caf	-	ts	-	wt, caf	-	wt	-	
		1	ts	-	ts	-	wt, caf	-	wt, caf	-	
195	F2 (I)	2	ts	-	ts	-	wt	-	wt	-	
		1	ts, caf	I	ts, caf	III	wt	-	wt	-	1
		1	ts, caf	I	ts, caf	II	wt	-	wt	-	1
		1	ts, caf	I	ts, caf	II	wt	-	wt	-	1
		1	ts	-	NG	-	wt	-	wt	-	
		2	ts, caf	-	wt, caf	-	wt	-	wt	-	
		1	ts	-	wt, caf	-	wt, caf	-	wt, caf	-	
		2	ts, caf	-	NG	-	NG	-	NG	-	
		1	ts, caf	-	ts	-	wt	-	wt	-	

Mutant	F Generation	Number of Zygospores	Phenotypes of progeny								Total Zygospores Tested in Liquid
			Progeny a		Progeny b		Progeny c		Progeny d		
			plate	liquid	plate	liquid	plate	liquid	plate	liquid	
92	F3 (I)	20	ts, caf	I	ts, caf	II	wt	-	wt	-	2
			ts, caf	II	ts, caf	III	wt	-	wt	-	1
			ts, caf	III	ts, caf	III	wt	-	wt	-	1
194	F3 (I)	3	ts, caf	I	ts, caf	III	wt	-	wt	-	1
			ts, caf	III	ts, caf	III	wt	-	wt	-	1
		2	ts, caf	III	ts	III	wt, caf	-	wt	-	1
		1	ts		ts		wt, caf	-	wt, caf	-	
		2	ts		ts		wt	-	wt	-	
195	F3 (I)	16	ts, caf	I	ts, caf	II	wt	-	wt	-	1
			ts, caf	III	ts, caf	III	wt	-	wt	-	1
		1	ts, caf	II	ts	II	wt	-	wt	-	1
	F3 (II)	14	ts, caf	II	ts, caf	II	wt	-	wt	-	2
		1	ts, caf	-	wt	-	wt	-	wt	-	
92	F4 (I)	20	ts, caf	I	ts, caf	III	wt	-	wt	-	3
			ts, caf	I	ts, caf	II	wt	-	wt	-	1
		1	ts, caf	II	ts, caf	III	wt	-	wt	-	1

Given the complex range of phenotypes observed it may allow more simple assimilation of the experimental data if the minimum number and type of mutation that can account for the phenotypes is proposed.

Two characters can be attributed to single independent genes. One is *cdc C* (cell division cycle cAMP related) conferring the character of division arrest (large cells blocked in division) on TAPYPP agar at 21°C. This property segregated 2:2 with wild-type among progeny from 74 zygospores of back crosses of mutant 92, and in 26 zygospores of back crosses of mutant 194 and 41 zygospores of back crosses of mutant 195. There is therefore clearly a single cdc gene in each mutant. The second single gene character (*caf R*) is resistance to 3 mM caffeine in TAPYPP agar at 21°C, and it also segregated 2:2 with wild-type among progeny from zygospores derived from back crosses in which the number of the zygospores analysed was 74 from mutant 92, 26 from mutant 194 and 41 from mutant 195. Although in minority of cases (Table 6.1; 195 F2 lines 7 and 9, and 195 F3 (II) line 2) the ratio of caffeine resistant : wt is not 2:2 this may be due to back mutation of the gene in occasional progeny.

Although all three original isolated mutants contained a *cdc C* and *caf R* type mutation, these were not in every case identical genes since in mutant 92 they were tightly linked giving zero recombinants among 296 progeny, and less tightly linked in mutant isolate 195 giving 12 recombinants from 164 progeny (7%) and even less tightly linked in mutant isolate 194, which gave 27 recombinants among 104 progeny (26%) with respect to *cdc C* and *caf R* in back crosses. Chi square (X^2) test can be applied to evaluate the possibility that the *cdc C* and *caf R* genes are significantly linked (Table 6.2).

Taking 3 degrees of freedom the probability is less than 0.005 that the observed ratio is a chance deviation resulting from unlinked genes. It is concluded that there is a better than 99.5% chance that the *cdc C* and *caf R* genes of mutant 92, 194 and 195 are linked. It is not possible to conclude from these data whether the *cdc C* genes or the *caf R* genes or both are different in these mutant isolates. This could be resolved by performing crosses between the different isolates or by forming diploids to test for complementation. This would be an early objective for continuation of this project.

Table 6.2 Chi square (X^2) test for linkage of *cdc C* and *caf R*; ts = *cdc C*, caf = *caf R*, df = degree of freedom

Mutant	Genotype	Observed frequency (O)	Expected frequency (E)	$(O-E)^2$	$(O-E)^2/E$
92	ts caf	148	74	5476	74
	ts +	0	74	5476	74
	+ caf	0	74	5476	74
	+ +	148	74	5476	74
		total = 296	total = 296		X^2 =296 df = 3 p < 0.005
194	ts caf	35	26	81	3.1
	ts +	17	26	81	3.1
	+ caf	10	26	256	9.8
	+ +	42	26	256	9.8
		total = 104	total = 104		X^2 =25.8 df = 3 p < 0.005
195	ts caf	71	41	900	21.9
	ts +	7	41	1156	28.2
	+ caf	5	41	1296	31.6
	+ +	81	41	1600	39
		total = 164	total = 164		X^2 =120.7 df = 3 p < 0.005

The characters, of different forms of division arrest and capacities for rescue by cAMP, are more difficult to analyse because they are clearly not determined by mutation of a single gene. For example cells with arrest type I when subject to further back cross can give rise to all of the phenotypic characters I, II, III, *cdc C* and *caf R*. Since this was invariably case with all of 141 zygospores analysed, then the type I liquid arrest phenotype can be attributed to the presence of all of the cAMP related genes that were affected by mutagenesis in *Chlamydomonas* during this study.

The type III phenotype, of no arrest in liquid although the presence of the *cdc C* gene is revealed by testing on agar, can be explained as due to the presence of the single *cdc C* mutation. Arrest in liquid due to this mutation must require the presence of additional mutations that alter the biochemical background, through effects on adenylate cyclase and cAMP level, so making the intracellular environment non permissive for function of the abnormal *cdc C* gene product. The physiological stress of growth on the surface of agar presumably establishes equivalent non permissive intracellular conditions in a normal genetic background. The greater physiological stress imposed by growth on the surface of agar and the capacity of such stress to result in failure of division, is repeatedly demonstrated during the attempted isolation of cdc mutants that will arrest in liquid. The replicate plating process for mutant isolation requires initial screening of colonies on agar but when those that have cdc properties on agar are tested further in liquid about 93% of them prove not to arrest in division in liquid although they do on agar (section III). This difference is not seen with yeast cells and it may correlate with a stress imposed by the illumination that is required to grow colonies of this photosynthetic organism. It takes ten days to form a colony from a single cell in bright light so lower light levels would be prohibitively slow. It is not clear what the functions of the *cdc C* type of gene is. The mutant form of *cdc C* that was present in each cAMP mutant isolate had the side effect of small colony size due to slow growth even at the permissive temperature of 21°C. The classic cdc gene is envisaged as not being involved with growth and solely affecting cell division.

There is some direct indication that the function of the *cdc C* gene is cAMP-related because in liquid medium, in which the malfunction is not sufficiently strong to block division at 33°C, the final stationary cell number is reduced compared with wild-type but can be increased to closer to the normal value by addition of cAMP giving final cell numbers at 33°C that are 1.5 fold higher than in unsupplemented medium at 33°C. However cellular cAMP levels, although lower than wild-type, remain appreciable in these type III cells at 33°C in liquid, unlike the very low or undetectable levels of cAMP in type I and II (section VIII, Table 8.1).

Two genes can be eliminated as not participating in the type III liquid phenotype. One is the *caf R* gene of 194 isolate. The *caf R* gene segregates separately from *cdc C* on occasions without affecting type III arrest (e.g. Table 6.1; 194 F2 line 4 and 194 F3 line 3). The other gene that is not involved in the type III liquid phenotype is the *res* gene

(described below) that is necessary for cAMP rescue. This gene has no detectable phenotype when present alone so it cannot be directly detected but the frequency of type III arrest would be far below the observed frequencies if it is dependent upon the combined presence of *cdc C* and *res* genes (described more fully below). The simplest explanation of type III phenotype is therefore that it derives simply from the presence of a *cdc C* gene. In all of the 36 cases of type III phenotype every one proved to have the *cdc C* phenotype when tested on agar (Table 6.1).

To account for the type II liquid arrest phenotype additional mutation(s) must be present. Type II cells clearly have a copy of the single *cdc C* genes that were present in the original isolates since they arrest on agar, but since they additionally arrest in liquid there must be an additional mutation that can be termed potentiating (*pot*). Enzyme assay indicated direct effects of this gene on adenylate cyclase activity (section VII; Table 7.4). The *cdc C* mutation that is revealed to be present in type II arresting cells by testing on agar does not have a strong effect on adenylate cyclase activity since activity remains high in cells of type III that have the *cdc C* mutation. The additional mutation (*pot)* present in type II greatly reduces this activity (section VII; Table 7.4). Cells of type II had adenylate cyclase activity that was less than 7% of wild-type cells. Type II cells of mutant 92 had no adenylate cyclase activity in the extract from cells grown at 21°C and assayed for the activity at 25°C and had 0.3 pmol/min/mg protein when assayed at 35°C. However extract of cells that had been arrested at 33°C for 24 h and assayed for activity at 35°C had no adenylate cyclase activity. Similarly in type II cells of mutant 195, cells grown at 21°C and assayed for activity at 25°C had adenylate cyclase activity at 0.3 pmol/min/mg protein but had no adenylate cyclase activity when assayed at 35°C, as did cells that had been arrested at 33°C for 24 h before assayed for the activity (section VII; Table 7.4).

The low activity of adenylate cyclase at 25°C of type II cells correlates with slower growth rate of these cells compared to cells of type III when cultured at 21°C. This is perhaps due to low cAMP production (section VIII; Table 8.1).

Low adenylate cyclase activity in type II cells correlates with a block to division in liquid medium at 33°C. The evidence for this comes from a comparison of adenylate cyclase activity of cell type II with type III. Cells of type III, although they showed arrest on agar plates due to the presence of the *cdc C* mutation, divided normally in liquid medium. Adenylate cyclase activity of type III cells which were cultured at 21°C and 33°C showed higher activity than in the other partial phenotypes I and II (section VII; Table 7.4). For example in partial phenotypes derived from mutant 92, cells type III cultured at 21°C and assayed activity at 25°C had adenylate cyclase activity of 1.73 unit which was 37% of the activity of wild-type cells and the activity at 35°C was 64 % of that of wild-type. Furthermore cells of type III from 92 that were cultured at 33°C for 24 h and assayed for activity at 35°C still retained 54% of the activity of wild-type cultured in the same condition. Similar results was obtained from mutant 194. Therefore the extra mutant gene *pot* that is present in type II cells had a strong effect in reducing of adenylate

cyclase activity at 33°C, compared with type III which shared *cdc C* but lacked *pot*, and at this temperature conditional loss of adenylate cyclase presumably resulted in the blocking of cell division.

The caffeine resistant mutation *caf R* is not necessary for the type II arrest phenotype since independent segregation of *caf R* from *cdc* produced, in the case of mutant 195, a type II arresting F3 cell that lacked *caf R* (e.g. Table 6.1; 195 F3 line 3). The lack of correlation of the *caf R* mutation with the type II phenotype is a little surprising since the selection protocol attempted to bias selection towards cells that, having become caffeine resistant, would presumably have a reduced cAMP synthesizing capacity. Therefore selecting among these for cAMP rescue would identifying cells that were depleted in cAMP when arrested. The properties of the mutants that were finally selected certainly correlate with reduced cAMP synthesizing capacity, but this was independent of the *caf R* gene as judged by the dispensibility of the *caf R* gene in cdc arrest.

To account for type I arrest at least one additional gene must be present in type I that is absent from type II arresting progeny. The additional gene allows rescue by cAMP since this property of rescue distinguishes these two phenotypes. The rescue gene (*res*) need not be a cAMP metabolizing gene, it could simply be a gene increasing permeability of the cell to cAMP. It would be interesting to make direct comparison of the rate of penetration of radioactive cAMP into the cells of type I and II to test this possibility. However there is direct evidence that the rescue gene has a moderating effect on the inactivation of adenylate cyclase by the *pot* gene mutation, so bringing cAMP levels into the range that can be supplemented to adequate intracellular levels by exogenous cAMP. This latter possibility is supported by adenylate cyclase activity data from both the mutants for which activity comparisons can be made in type I and type II partial phenotypes. In isolate 92, type I cells had adenylate cyclase activity of 1.8 pmol/mg protein in the extract from cells grown at 21°C when assayed at 25°C compared with undetectable activity from type II cells lacking the rescue gene when grown and assayed at the same temperature (section VII; Table 7.4). Therefore presence of the *res* gene in type I increased adenylate cyclase activity in comparison with type II that lacked the *res* gene. However extracts from cells that had been arrested at 33°C for 24 h when derived from this mutant 92 had no detectable adenylate cyclase measured at 35°C whether of type I or type II after back crossing. Therefore no effect of the rescue gene on basal level was detectable by this comparison. Similarly in isolate 195 extracts from cells at 21°C of type I or type II when assayed at 25°C had adenylate cyclase activity of 1.3 and 0.3 pmol/mg protein respectively and extracts from cells of these two types grown at the same temperature and assayed for the activity at 35°C, had adenylate cyclase activity of 1.2 pmol/mg protein and not detectable respectively, showing a consistently higher activity correlating with presence of the rescue gene that differentiates type I cells from type II. The only exception to this correlation is in extracts of 92 taken from cells grown at 21°C and

assayed at 35°C, in which levels of adenylate cyclase activity were low in both type I and II being zero and 0.3 units respectively. The lower activity from type I cells does not conform to the postulated positive effect from the *res* gene, but the significance of differences between these levels, both of which are less than 7% of normal is not clear. The suggestion that the *res* gene preserves adenylate cyclase activity is supported by the evidence that cAMP levels are higher in cells of type I at 21°C and 33°C than in cells of type II that lack the capacity to be rescued by exogenous cAMP (sectionVIII; Table 8.1).

The minimum number of mutant genes that was present in each of the original isolates, and was recovered in each of the progeny of back crossing that had retained the phenotype of type I arrest in liquid is therefore the following;

1. *cdc C* gene; mutation in this gene was sufficient to cause division arrest at 33°C under the culture conditions prevailing on illuminated agar plates. It caused slow growth and small colony size, and also reduced stationary cell density in liquid medium unless supplemented with cAMP.

2. *pot* gene; mutation in this gene enhanced the effect of the *cdc C* gene and gave arrest in liquid medium at 33°C. Presence of mutant genes of this type correlated with reduced activity and with *in vitro* thermolability of adenylate cyclase. In view of the latter observation it may encode a protein directly concerned in adenylate cyclase catalytic activity.

3. *res* gene; mutation in this gene allows cells that contain the *cdc C* gene and are presumed to contain the *pot* gene to resume cell division in liquid medium at 33°C if supplemented with cAMP. The *res* gene mutation raises the basal level of adenylate cyclase activity in cells containing the *pot* mutation and this may explain its promotion of rescue.

4. *caf R* gene; mutation in this gene results in caffeine resistance at 21°C. It does not appear to be essential for the cdc phenotype on agar in the case of 194 type III (Table 6.1; 194 F2 line 4 and F3 line 3) in which it can segregate independently from division arrest. It is not essential for the liquid cdc phenotype in the case of 195 type II (Table 6.1; 195 F3 line 3).

The combination of genes that are hypothesised to give the arrest phenotypes observed are given below. The possibility that these combinations account for the phenotypes was tested by predicting the frequency with which phenotypes would be observed among progeny if the hypothesised gene combinations were operative in giving the phenotypes.

Type III; genotype : *cdc C*

phenotype: cells arrest on agar plates at 33°C but can divide in liquid medium at 33°C, *caf R* is often present because of chromosomal linkage but occasional independent segregation indicates non participation of *caf R* in cdc arrest. The possible requirement of the *res* gene in contributing to this phenotype (which seemed possible

because of the higher final cell number that was obtained when medium was supplemented with cAMP) was tested and was rejected by predicting phenotype frequency according to Table 6.3 below.

Type II; genotype : *cdc C+pot*

phenotype: cells arrest on agar plates at 33°C and in liquid medium at 33°C but are not rescued with respect to division by added cAMP. The *caf R* gene is often present because it is linked to *cdc C* but independent segregation in 195 has shown that *caf R* is not necessary for liquid cdc arrest.

Type I; genotype : *cdc C+pot+res*

phenotype: cells arrest on agar plates at 33°C and in liquid medium at 33°C and they are rescued by cAMP or db-cAMP (but not AMP) to division at 33°C. The *caf R* mutation was present in all type I cells that have been tested, but this might be due to chance and it may not indicate that it is essential for type I phenotype because it is clearly unnecessary for liquid cdc arrest in type II and type III.

The validity of the hypothesis that the three phenotypes seen in liquid medium are due to the different combinations of the three genes, *cdc C, pot* and *res* can be tested in two ways, which both depend upon comparing the frequency of occurrence of the phenotypes with the frequency with which the gene combinations would be expected to occur according to Mendelian geneties upon back crossing. One comparison can be made by predicting gross frequencies in the total progeny, ignoring their occurrence in tetrads. A second comparison can be made using the data from individual tetrads in comparison with the predicted frequency of co-occurrence of phenotypes within single tetrads.

Considering phenotype frequency among total progeny, without regard to frequency in individual tetrads, the following considerations apply to phenotypes arising from a back cross of

$cdc\ C^-\ pot^-\ res^-$ x $cdc\ C^+\ pot^+\ res^+$

which can be abbreviated as :

c p r x + + +

Making the assumption of minimum complexity that these are unlinked genes, which is probable in view of the estimate that there are 16-18 chromosomes in *C.*

reinhardtii (reviewed by Harris, 1988), and therefore that the genes can segregate independently at the first meiotic division, the following combinations are expected:-

Combination	Predicted phenotype
c p r + + +	I wt
+ p r c + +	wt III
c + r + p +	III wt
c p + + + r	II wt

In this table, the phenotypes are predicted on the basis that the *cdc C* gene alone is capable of giving the type III phenotype. Chi square (X^2) test can be applied to evaluate this possibility (Table 6.3).

Table 6.3 X^2 test for the phenotypic prediction based on the assumption that the type III phenotype derives from the presence of *cdc C* gene alone.

Phenotype	Probability	Observed frequency (O)	Expected frequency (E)	$(O-E)^2$	$(O-E)^2/E$
I	0.125	21	18	9	0.5
II	0.125	16	18	4	0.22
III	0.25	36	36	0	0
wt	0.5	71	72	1	0.014
		total = 144	total = 144		$X^2 = 0.734$ df = 3 p = 0.87

Taking 3 degrees of freedom, the probability that the deviation of the observed result from the expected result is entirely due to the chance deviation is 0.87, which is

comfortably above the threshold of 0.05 and therefore supports the postulation that the type III phenotype derives simply from the presence of *cdc C*gene.

This form of analysis also allows the rejection of the possibility that the type III phenotype required the combined presence of both *cdc C* and *res* mutations. The predicted frequencies in that case would be:

Table 6.4 X^2 test for the phenotypic prediction based on the assumption that the type III phenotype derives from the combined presence of both *cdc C* and *res* genes.

Phenotype	Probability	Observed frequency (O)	Expected frequency (E)	$(O-E)^2$	$(O-E)^2/E$
I	0.125	21	18	9	0.5
II	0.125	16	18	4	0.22
III	0.125	36	18	324	18
wt	0.625	71	90	361	4
		total =144	total = 144		$X^2 = 22.77$ df = 3 $p < 0.005$

Clearly the probability that the hypothesis is true that the deviation is due to statistical sampling error is far below the acceptable limits of significance and therefore the hypothesis that *cdc C* and *res* are both required for type III arrest can be rejected.

A more rigorous analysis is possible if individual tetrads are considered because it is then possible to investigate whether the genes are tightly linked to the centromeres, which would prevent crossing over at the four strand stage and therefore limit the number of possible new partial phenotypes that could be obtained from three genes within a single tetrad, specifically without such recombination between gene and centromere it is only possible to account for tetrads of wt:wt:I:I, wt:wt:III:III and wt:wt:II:II but not wt:wt:I:III or wt:wt:II:III. Investigation of the tetrads also provides a test of whether (i) recombination of the three genes and their postulated contribution to the phenotypes can

indeed simply predict the particular grouping of phenotypes seen within the products of individual meiotic divisions and (ii) whether these predicted frequencies accurately conform to the observed frequencies.

To predict the possible new combinations of genes that can arise on back crossing of these mutants to wild-type it must be recognized that new combinations of unlinked genes result from two processes. One process is the independent segregation of homozygous pairs of chromosomes at first meiosis. The other process allowing new gene combinations is recombination between gene and centromere occurring at the four strand stage of the first meiotic division.

Operation of independent segregation allows that when one member of an homologous pair, travels to a spindle pole it may or may be joined, entirely due to chance, by either of the chromosomes of each other homologous pair. In the case of the three genes that affect the cAMP division phenotypes of *Chlamydomonas* this independent segregation is predicted to produce partial phenotypes due to combinations including some but not all the three mutant genes.

The possible combinations of three unlinked genes that are not adjacent to their centromere, which can occur among the progeny of a single zygospore by Mendelian segregation, are summarised in Table 6.5. The derivation of this table is simple but will probably not be intuitively obvious to readers who are not currently involved in Mendelian genetic analysis. Therefore, to explain the derivation of the summary table, the effects of zero, single, double and triple recombination events on gene segregation patterns at meiosis are illustrated in the next eight tables. In connection with each of these tables it should be borne in mind that the crossing over during first meiosis are illustrated in Table 6.5. After first meiosis each nucleus contains only one chromosome from each homologous pair and the chromosomes at that stage contain two chromatids. After second meiosis each chromosome contains a single chromatid.

This can be indicated diagrammatically as follows:-

a. No crossing over between gene and centromere results in the following possible segregation, which are summarised in the top row of Table 6.5.

possible nuclei [ ] formed by first meiosis	Nuclei [ ] after second meiosis	Phenotypes of the four progeny of a single zygospore
[c p r]	[c p r]	I
[c p r]	[c p r]	I
[+ + +]	[+ + +]	wt
[+ + +]	[+ + +]	wt
[c p +]	[c p +]	II
[c p +]	[c p +]	II
[+ + r]	[+ + r]	wt
[+ + r]	[+ + r]	wt
[c + r]	[c + r]	III
[c + r]	[c + r]	III
[+ p +]	[+ p +]	wt
[+ p +]	[+ p +]	wt
[c + +]	[c + +]	III
[c + +]	[c + +]	III
[+ p r]	[+ p r]	wt
[+ p r]	[+ p r]	wt

The diversity of phenotypes within a single zygospore that can be generated by independent segregation is limited by the fact that at the second meiotic division, when the chromatids separate, only identical pairs of nuclei can be derived from each of the haploid nuclei formed at first meiosis. Consequently phenotypes will occur in identical pairs in individual zygospores if the only force for resortment is independent segregation.

A diversity of phenotypes within a single zygospore is possible if recombination occurs between gene and centromere at the first meiotic division. Such recombination is only unlikely in the case of the minority of genes that are located close to the centromere. Most genes will be affected by such recombination and there is direct evidence, from the occurrence here of mixed phenotypes I+III and II+III in individual zygospores, that recombination occurs between centromeres and the cAMP-division genes.

The effect of crossing over between centromere and mutant gene can be illustrated as follows:-

b. A single crossing over between centromere and the *cdc C* gene results in the following possible segregations, which are summarised in the second row of Table 6.5.

possible nuclei [ ] formed by first meiosis	Nuclei [ ] after second meiosis	Phenotypes of the four progeny of a single zygospore
[c p r]	[c p r]	I
[+ p r]	[+ p r]	wt
[c + +]	[c + +]	III
[+ + +]	[+ + +]	wt
[c p +]	[c p +]	II
[+ p +]	[+ p +]	wt
[c + r]	[c + r]	III
[+ + r]	[+ + r]	wt
[+ p r]	[+ p r]	wt
[c p r]	[c p r]	I
[+ + +]	[+ + +]	wt
[c + +]	[c + +]	III
[+ p +]	[+ p +]	wt
[c p +]	[c p +]	II
[+ + r]	[+ + r]	wt
[c + r]	[c + r]	III

c. A single crossing over between centromere and the *pot* gene results in the following possible segregations, which are summarised in the third row of Table 6.5.

possible nuclei [ ] formed by first meiosis	Nuclei [ ] after second meiosis	Phenotypes of the four progeny of a single zygospore
$\overline{c}\ p\ \overline{r}$	[c p r]	I
$\underline{c}\ +\ \underline{r}$	[c + r]	III
$\overline{+}\ p\ \overline{+}$	[+ p +]	wt
$\underline{+}\ +\ \underline{+}$	[+ + +]	wt
$\overline{c}\ p\ \overline{+}$	[c p +]	II
$\underline{c}\ +\ \underline{+}$	[c + +]	III
$\overline{+}\ p\ \overline{r}$	[+ p r]	wt
$\underline{+}\ +\ \underline{r}$	[+ + r]	wt
$\overline{c}\ +\ \overline{r}$	[c + r]	III
$\underline{c}\ p\ \underline{r}$	[c p r]	I
$\overline{+}\ +\ \overline{+}$	[+ + +]	wt
$\underline{+}\ p\ \underline{+}$	[+ p +]	wt
$\overline{c}\ +\ \overline{+}$	[c + +]	III
$\underline{c}\ p\ \underline{+}$	[c p +]	II
$\overline{+}\ +\ \overline{r}$	[+ + r]	wt
$\underline{+}\ p\ \underline{r}$	[+ p r]	wt

d. A single crossing over between centromere and the *res* gene results in the following segregations which are summarised in the fourth row of Table 6.5.

possible nuclei [ ] formed by first meiosis	Nuclei [ ] after second meiosis	Phenotypes of the four progeny of a single zygospore
[c p r]	[c p r]	I
[c p +]	[c p +]	II
[+ + r]	[+ + r]	wt
[+ + +]	[+ + +]	wt
[c + r]	[c + r]	III
[c + +]	[c + +]	III
[+ p r]	[+ p r]	wt
[+ p +]	[+ p +]	wt
[c p +]	[c p +]	II
[c p r]	[c p r]	I
[+ + +]	[+ + +]	wt
[+ + r]	[+ + r]	wt
[c + +]	[c + +]	III
[c + r]	[c + r]	III
[+ p +]	[+ p +]	wt
[+ p r]	[+ p r]	wt

e. Double crossing overs between centromeres and the *cdc C* and the *res* genes. These sets of progeny is summarised in the fifth row of Table 6.5.

possible nuclei [ ] formed by first meiosis	Nuclei [ ] after second meiosis	Phenotypes of the four progeny of a single zygospore
$\overline{[c\ p\ r]}$	[c p r]	I
$\underline{[+\ p\ +]}$	[+ p +]	wt
$\overline{[c\ +\ r]}$	[c + r]	III
$\underline{[+\ +\ +]}$	[+ + +]	wt
$\overline{[c\ +\ r]}$	[c + r]	III
$\underline{[+\ +\ +]}$	[+ + +]	wt
$\overline{[c\ p\ r]}$	[c p r]	I
$\underline{[+\ p\ +]}$	[+ p +]	wt
$\overline{[c\ p\ +]}$	[c p +]	II
$\underline{[+\ p\ r]}$	[+ p r]	wt
$\overline{[c\ +\ +]}$	[c + +]	III
$\underline{[+\ +\ r]}$	[+ + r]	wt
$\overline{[c\ +\ +]}$	[c + +]	III
$\underline{[+\ +\ r]}$	[+ + r]	wt
$\overline{[c\ p\ +]}$	[c p +]	II
$\underline{[+\ p\ r]}$	[+ p r]	wt

f. Double crossing overs between centromeres and the *cdc C* and the *pot* genes. These set of progeny are summarised in the sixth row of Table 6.5.

possible nuclei [ ] formed by first meiosis	Nuclei [ ] after second meiosis	Phenotypes of the four progeny of a single zygospore
[c p r]	[c p r]	I
[+ + r]	[+ + r]	wt
[c p +]	[c p +]	II
[+ + +]	[+ + +]	wt
[c p +]	[c p +]	II
[+ + +]	[+ + +]	wt
[c p r]	[c p r]	I
[+ + r]	[+ + r]	wt
[c + r]	[c + r]	III
[+ p r]	[+ p r]	wt
[c + +]	[c + +]	III
[+ p +]	[+ p +]	wt
[c + +]	[c + +]	III
[+ p +]	[+ p +]	wt
[c + r]	[c + r]	III
[+ p r]	[+ p r]	wt

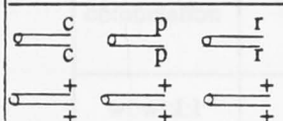
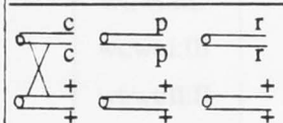
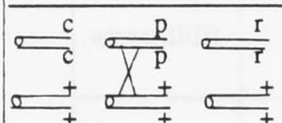
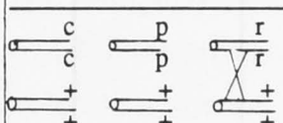
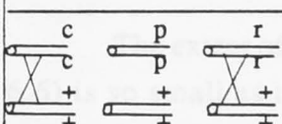
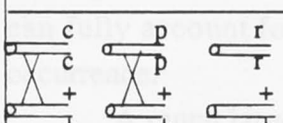
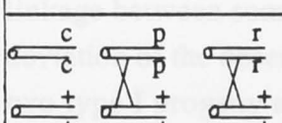
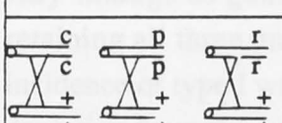
g. Double crossing overs between centromeres and the *pot* and the *res* genes. These set of progeny are summarised in the seventh row of Table 6.5.

possible nuclei [ ] formed by first meiosis	Nuclei [ ] after second meiosis	Phenotypes of the four progeny of a single zygospore
c p r c + +	[c p r] [c + +]	I III
+ p r + + +	[+ p r] [+ + +]	wt wt
+ p r + + +	[+ p r] [+ + +]	wt wt
c p r c + +	[c p r] [c + +]	I III
c p + c + r	[c p +] [c + r]	II III
+ p + + + r	[+ p +] [+ + r]	wt wt
+ p + + + r	[+ p +] [+ + r]	wt wt
c p + c + r	[c p +] [c + r]	II III

h. Triple crossing overs between centromeres and the *cdc C, pot, res* genes, results in following segregation, which are summarised in the eighth row of Table 6.5.

possible nuclei [ ] formed by first meiosis	Nuclei [ ] after second meiosis	Phenotypes of the four progeny of a single zygospore
[c p r]	[c p r]	I
[+ + +]	[+ + +]	wt
[c p r]	[c p r]	I
[+ + +]	[+ + +]	wt
[c p +]	[c p +]	II
[+ + r]	[+ + r]	wt
[c p +]	[c p +]	II
[+ + r]	[+ + r]	wt
[c + r]	[c + r]	III
[+ p +]	[+ p +]	wt
[c + r]	[c + r]	III
[+ p +]	[+ p +]	wt
[c + +]	[c + +]	III
[+ p r]	[+ p r]	wt
[c + +]	[c + +]	III
[+ p r]	[+ p r]	wt

Table 6.5 Summary of the expected frequencies of phenotype derives from zero, single, double and triple recombination events of three unlinked genes that are not adjacent to their centromere.

Recombination between gene and centromere	Frequencies of sets of offspring in individual zygospores					
	wt:wt:I:I	wt:wt:I:II	wt:wt:I:III	wt:wt:II:II	wt:wt:II:III	wt:wt:III:III
	1	-	-	1	-	2
	-	1	1	-	2	-
	-	-	2	-	1	1
	-	2	-	-	-	2
	-	-	2	-	2	-
	-	2	-	-	2	2
	-	-	2	-	2	-
	1	-	-	1	-	2
Total expected	2	5	7	2	7	9
Observed number	0	5	7	3	3	9

The predicted frequencies of phenotypic combinations in single zygospores, resulting from zero, single, double and triple recombination events between the unlinked genes and their centromeres can be evaluated using the X^2 test as shown in Table 6.6.

Table 6.6 X^2 test for the predicted frequency of mixed phenotypes in single tetrads.

Possible combination	Probability	Observed frequency(O)	Expected frequency(E)	$(O-E)^2$	$(O-E)^2/E$
wt:wt:I:I	2/32	0	1.7	2.89	1.7
wt:wt:I:II	5/32	5	4.2	0.64	0.15
wt:wt:I:III	7/32	7	5.9	1.21	0.2
wt:wt:II:II	2/32	3	1.7	1.69	0.99
wt:wt:II:III	7/32	3	5.9	8.41	1.42
wt:wt:III:III	9/32	9	7.6	1.96	0.26
	total = 27	total = 27			$X^2 = 4.72$ df = 5 p = 0.47

The extent of deviation of the observed result from that predicted (shown in Table 6.6) is so small as to be easily accounted for by sample variability. Therefore the assumptions that the three genes are neither linked to each other nor to the centromeres can fully account for the nature of the partial phenotypes and the frequency of their occurrence.

A much larger sample would be necessary to eliminate the possibility of slight linkage between some of the genes but there is no evidence of linkage. Indeed the main deviation of the observed data from the predicted is a lower incidence of zygospores with two type I progeny and a higher incidence of those with type II together with type III. Any linkage of genes would be expected to result in a higher incidence of progeny retaining all three mutations and hence higher incidence of type I arrest, whereas lower incidence of type I was observed. Therefore absence of linkage is highly possible and any deviation from the expected result is probably due to imperfect representation of the true result by the small sample taken for observation. All forms of analysis, therefore, correlate with the hypothesis that three genes interact in the manner postulated in affecting the contribution of cAMP to cell division in *Chlamydomonas*.

SECTION VII ENZYME ACTIVITIES OF ts-cAMP REQUIRING MUTANTS

Cyclic AMP is synthesized from ATP by adenylate cyclase (Jost and Rickenberg, 1974) and is hydrolysed to 5'AMP by phosphodiesterase (Cheung, 1970). Since the three mutations isolated here were rescued by cAMP supplementation it was considered possible that the mutations caused a reduction of intracellular levels of cAMP. To investigate the possible biochemical basis of this defect attention was focussed on two enzymes; the biosynthetic enzyme adenylate cyclase, which might have been reduced in activity, and the degradative enzyme phosphodiesterase, which might have been increased in activity. Back crossing of the original isolated mutants indicated that four genes contributed to their phenotypes. Segregation of the contributing genes in repeated back crossing gave the original and two partial phenotypes; type I progeny retained the originally isolated phenotype of ts-cdc arrest in liquid with rescue by cAMP or db-cAMP; type II retained ts-cdc arrest in liquid without capacity for rescue; type III showed cdc arrest on agar only, not in liquid, but were stimulated to slightly higher stationary cell density when supplemented with cAMP and 5'-AMP. The genetic basis of these phenotypes is discussed in section VI. Cells with these three different phenotypes after repeated back crossing were investigated by enzyme assay to determine the level and thermosensitivity of both phosphodiesterase and adenylate cyclase at 25°C and 35°C.

Cyclic 3',5'-nucleotide phosphodiesterase activity in *C. reinhardtii* was first reported by Fischer and Amrhein (1973). The activity that they detected resembled in many aspects the cyclic nucleotide phosphodiesterases that have been described in animals (Yamamoto, 1969; Cheung, 1970), bacteria (Branna and Chytil,1966; Kobayashi, 1970) slime mould (Chang, 1968), fungi and protozoans (Blum, 1970; Ramanathan, 1973) rather than those found in higher plants. For instance the pH optimum of the *Chlamydomonas* enzyme *in vitro* is reported by Fischer and Amrhein (1973) to be in the alkaline range (pH 8.5) and it requires the presence of a sulfhydryl agent, such as 2-mercaptoethanol, dithiothreitol or cysteine, and a divalent cation, Mg^{2+} or Mn^{2+}. The enzyme activity is inhibited by methylxanthine, papavarine, inorganic phosphate, ATP, Zn^{2+} and Co^{2+} but imidazole stimulates activity. *Chlamydomonas* phosphodiesterase hydrolyses cAMP at the 3'-phosphate ester bond and gives rise to 5'-AMP as the reaction product (Fischer and Amrhien, 1973). Higher plant cyclic nucleotide phosphodiesterases are reported to be

more active toward 2',3'-cAMP and usually generate two reaction products, predominantly 3'-AMP, with lesser amount of 5'-AMP (Niles and Mount, 1974; Junker et.al.,1977, Smeltzer and Johnson, 1977; Brown et.al.,1977). Furthermore, the higher plant enzymes are insensitive to methylxanthines and divalent metal ions (Robinson, et.al.,1971; Thompson and Appleman, 1971; Lin and Varner, 1972; Giannattasia, et.al., 1974, Ashton and Polya, 1975). However, cyclic nucleotide phosphodiesterase in *Phaseolus vulgaris* shows some similarity to animal phosphodiesterase in that it has a neutral pH optimum, produces 5'-AMP as a major end product and is inhibited by methylxanthine and imidazole (Brown et al., 1975, 1977; Dupon et al., 1987).

The cyclic nucleotide phosphodiesterase activity observed in this study confirmed the characteristics reported previously for the *Chlamydomonas*. Table 7.1 shows that the activity of phosphodiesterase in the presence of 1 mM Ca^{2+} and 5 mM Mg^{2+} at pH 7.5 was higher than at pH 6.2. The activity was significantly dependent upon calcium since omitting Ca^{2+} and including EDTA which removed residual free Ca^{2+} reduced activity by 22%. However the presence of calmodulin in addition to Ca^{2+} did not cause higher activity indicating either independence from calmodulin or presence of a saturating amount of the calcium binding protein in crude extracts. Activity of the enzyme, in the presence of 1 mM Ca^{2+}, 5 mM Mg^{2+} and calmodulin, was inhibited 76% by the presence of 5 mM theophylline and 50% by the presence of 5 mM caffeine.

The adenylate cyclase activity measured in this study probably closely resembles the activity studied by Hintermann and Parish (1979) who also used whole cell extracts of *C. reinhardtii* vegetative cells. The enzyme studied here was extracted by breaking cells with glass beads and activity in the presence of 10 mM Mg^{2+} at pH 7.5 was not significantly affected by the presence of 100 μM GTP while lowering to pH 6.2 in the presence of Mg^{2+} reduced activity (Table 7.2). The activity was low in the absence of Mg^{2+}. The activity after freezing was nil which indicated that the enzyme is unstable. The identity of the cyclic nucleotide produced by adenylate cyclase activity was confirmed by adding phosphodiesterase which reduced adenylate cyclase activity (Table 7.2).

The adenylate cyclase measured in this study may well be different from that investigated by Pasquale and Goodenough (1987), who measured only the enzyme that is present in the flagellar membrane of *Chlamydomonas* gamete cells. Gametic cells do not divide but use cAMP during mating, which promotes flagellar adhesion, during mating.

The flagellar enzyme was assayed in the presence of free calcium and was inhibited by the calmodulin antagonist, trifluoperazine. The activity measured in the present investigation was assayed in the presence of 0.1 mM EDTA and 0.1 mM EGTA and is therefore calcium independent whereas the activity described by Pasquale and Goodenough had available free calcium and was inhibited by trifluoperazine, was preferentially stimulated by Mn^{2+}. It was unresponsive to G-protein activators such as non hydrolysable GTP ($GTP_{\gamma}S$), AlF_4^- and forskolin and is therefore probably not associated with the G-protein. Similar properties of adenylate cyclase have been found in the enzymes of cilia from animal such as *Trypanosoma cruzi* (Torruela et al.,1986) molluscs (Kopf, 1984) and mammalian sperm (Hildelbrandt, et al., 1985). Pasquale and Goodenough concluded that the bulk of the activity that they measured was located in the flagellar membrane and was particularly abundunt in the flagellar membranes of the gametes. However a recent study by Kooijiman et al. (1990) concluded that in *C. eugametos* gamete cells the bulk of the adenylate cyclase activity is located in the membrane fraction of the cell body.

Since cell division occurs in the body of the cell it was judged more appropriate to test for possible modification to adenylate cyclase activity in total cell extract rather in the subfraction that may be present in the flagellar membrane. It is also relevant that although gamete flagellar membranes contain significant adenylate cyclase this may correlate with the significance of cAMP in mediating flagellar adhesion in mating. Cell division does not occur in gametes and in the vegetative cells that do divide the bulk of adenylate cyclase activity is located in the body of the cell, at least in *Chlamydomonas eugametos* (Kooijiman et al., 1990), therefore total adenylate cyclase was measured here.

Table 7.1 Investigation of assay conditions influencing activity of phosphodiesterase in whole cell extract of wild-type cells.

Histograms were derived from the data in Table 7.1

CaM = calmodulin ; caff = caffeine ; theo = theophylline

Phosphodiesterase activity was assayed with 2 mM DTT, 60 μg BSA, 5 mM $MgCl_2$, 20 μM cAMP, 30,000 cpm [^{3}H]cAMP (specific activity 41.7 Ci/mmol) and with the modification as indicated. The reaction was started by addition of 20-50 μg protein of crude cell extracts to mixtures prewarmed to 25°C and continued for 15 min until terminated by immersion in a boiling water bath for 2 min. After the sample was cooled, 20 μl of nucleotidase containing snake venom (2 mg/ml) was added and incubated for 10 min at 30°C. One ml of a mixture of resin : methanol (1:3) was added, mixed and left on ice for 5 min. The suspension was then centrifuged (3000 rpm for 10 min) and supernatant was used to count for ^{3}H.

Table 7.1 Phosphodiesterase activity of wild-type cells .

Assay condition	pmol/min/mg protein
pH 7.5 + 1 mM Ca^{2+}	684
pH 7.5 + 1 mM Ca^{2+} + 1 µg CaM	657
pH 7.5 + 1 mM EDTA	533
pH 6.2 + 1 mM Ca^{2+} + 1 µg CaM	250
pH 7.5 + 5 mM caff + 1 mM Ca^{2+} + 1 µg CaM	338
pH 7.5 + 5 mM theo + 1 M Ca^{2+} + 1 µg CaM	167

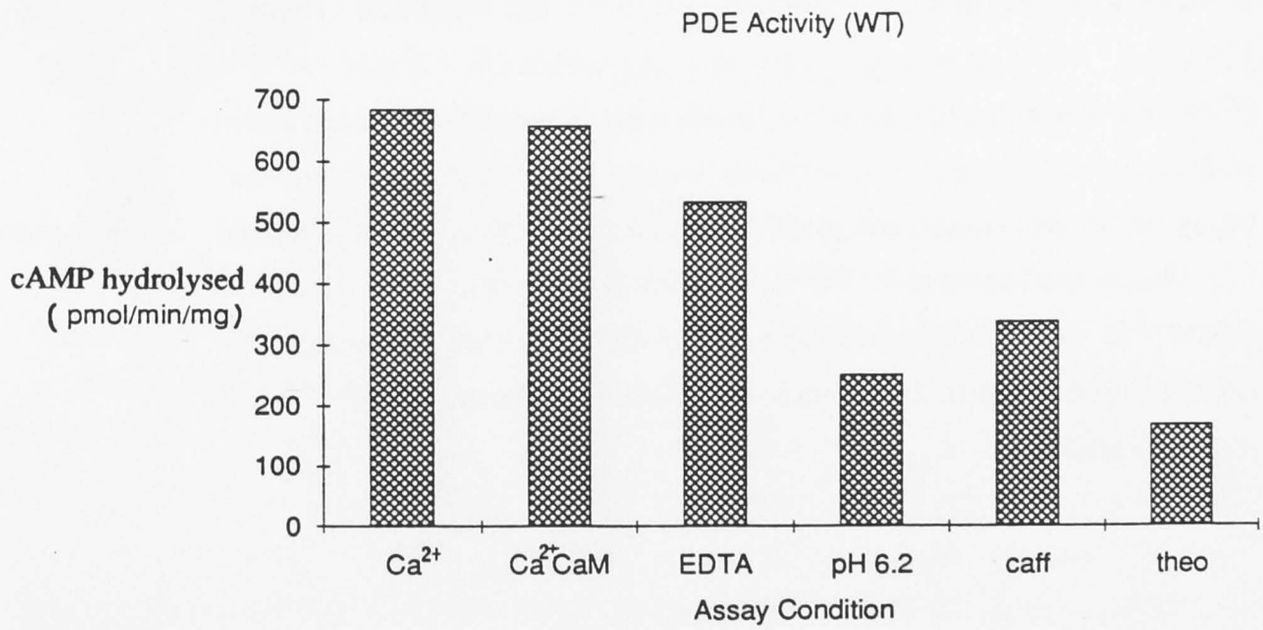


Table 7.2 Investigation of optimum conditions for assay of adenylate cyclase activity in crude whole cell extract of wild-type cells.

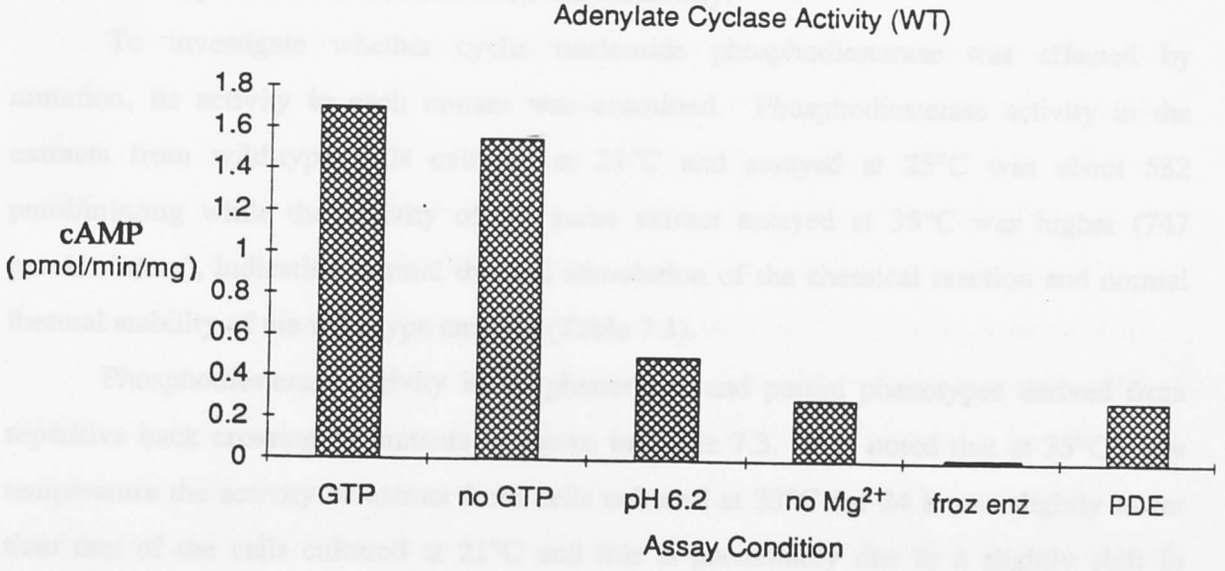
Histograms were derived from data in the Table 7.2.

PDE = phosphodiesterase

Adenylate cyclase activity was assayed with 300 μM cAMP, 0.1 mg/ml BSA, 0.1 mM EGTA, 0.1 mM EDTA, 20 mM creatine phosphate, 400 μg/ml creatine kinase, 1 mM DTT, 5 mM caffeine, 0.25 mM ATP, 1 x 10^6 cpm [^{32}P]ATP (specific activity 3000 Ci/mmol) and with the modifications as indicated. The reaction was started by adding 50 μg protein of crude cell extracts to mixtures prewarmed to 25°C, continued for 10 min and terminated by adding 100 μl of "stopping solution" (2% SDS, 45 mM ATP, 1.3 mM cAMP). [^{32}P]cAMP was isolated by decanting sample into a Dowex-50 column and eluted with 20 ml distilled water. Pooled fractions containing [^{32}P]cAMP were passed through an alumina column and cAMP was eluted with 0.1 M imidazole buffer pH 7.3. Eluates were counted for ^{32}P and ^{3}H. Phosphodiesterase (Boehringer) at 1.6×10^{-3} U was added to the mixture after adenylate cyclase reaction was terminated and incubated for 15 min at 37°C in the presence of 1 mM Ca^{2+} and 1 μM sheep brain CaM.

Table 7.2 Adenylate cyclase activity of wild-type cells

Assay condition	pmol/min/mg protein
pH 7.5 + 100 μM GTP + 10 mM Mg^{2+}	1.7
pH 7.5 no GTP + 10 mM Mg^{2+}	1.55
pH 6.2 + 10 mM Mg^{2+}	0.5
pH 7.5 no Mg^{2+}	0.3
pH 7.5 (frozen enzyme)	0.0
pH 7.5 + PDE	0.3



Phosphodiesterase and adenylate cyclase activities in wild- type and mutants

The capacity of the mutants to be rescued by cAMP or db- cAMP suggested that they may be unable to produce or accumulate enough cAMP. It is possible that these mutants have reduced adenylate cyclase activity or increased phosphodiesterase activity or both. The mutations may directly alter these enzyme proteins or may indirectly modulate the level or activity of one or both enzymes.

The mutants were selected for ability to divide at 21°C but not at 33°C, however, enzyme assays were conducted at 25°C and 35°C. The assay temperatures were selected for the following reasons. The 25°C temperature allows good activity and does not approach the nonpermissive temperature. The 35°C temperature is above the nonpermissive temperature for these mutants although it is certainly not above the temperature range of *Chlamydomonas* wild-type cells and therefore normal enzyme proteins should be unaffected. In the case of phosphodiesterase and adenylate cyclase in particular other workers have reported that 35°C is suitable for assay of the normal enzyme (Hintermann, 1979; Pasquale and Goodenough, 1987) and this temperature was therefore adopted as a useful test for possible mutation-induced thermolability.

To investigate whether cyclic nucleotide phosphodiesterase was affected by mutation, its activity in each mutant was examined. Phosphodiesterase activity in the extracts from wild-type cells cultured at 21°C and assayed at 25°C was about 532 pmol/min/mg while the activity of the same extract assayed at 35°C was higher (747 pmol/min/mg), indicating normal thermal stimulation of the chemical reaction and normal thermal stability of the wild-type enzyme (Table 7.3).

Phosphodiesterase activity in all phenotypes and partial phenotypes derived from repetitive back crossing of mutants is shown in Table 7.3. It is noted that at 35°C assay temperature the activity of extract from cells cultured at 33°C for 24 h was slightly lower than that of the cells cultured at 21°C and this is presumably due to a slightly shift in proportion of enzymes in cells grown at different temperature. There was no significant difference in level, or in heat stability when incubated at 25°C and 35°C, of activities from mutants in comparison with the activity of the wild type strain 125^{+}. Since there is no difference in level or stability of phosphodiesterase, attention was then focussed on adenylate cyclase as a more likely explanation of the cAMP related ts-cdc phenotypes.

Adenylate cyclase activity of all three mutants differed from wild-type (Table 7.4). Assay of the phenotypes obtained after repeated back crossing showed that the property of arrest in liquid at 33°C in both phenotypes I and II, derived by repeated back crossing to wild-type of these mutants, correlated with activity measured *in vitro* at 35°C that was less than 7% of the wild-type activity from normal cells at the same growth and assay temperatures. Similarly cells of type I and II after arrest at 33°C had adenylate cyclase activity less than 5% of the activity of wild-type. Thus the biochemical basis for the ts-cdc phenotype can be suggested in general terms, as involving altered adenylate cyclase activity.

More detailed analysis of the adenylate cyclase activity in the three different phenotypes that resulted from different gene combinations generated by back crossing, corroborates this interpretation but indicates differences in effect on adenylate cyclase between the several types of mutant gene that were present in each of the three originally isolated mutants. Corroboration of low adenylate cyclase activity contributing to the ts-cdc arrest in liquid comes from the much higher activity of type III progeny, which do not arrest in liquid, compared with the activity in type I and type II, which do arrest. In type III progeny derived from mutant 92, adenylate cyclase activity of cells cultured at 21°C and assayed for activity at 35°C showed a nine-fold higher activity than cells of type II cultured at 21°C and assayed for activity at 35°C. The activity of cell type III cultured at 33°C for 24 h and assayed for activity at 35°C was even higher while cells of type II after arrested at 33°C for 24 h had no detectable activity. This correlated with the lack of ts-cdc arrest in liquid by the type III progeny, in contrast with type II which did arrest in liquid.

A simple model of an adenylate cyclase that is active at 25°C but inactivated at 35°C is consistent with the data from type I arresting progeny after repeated backcross of mutant 92. These progeny showed 1.8 pmol/min/mg protein of adenylate cyclase activity when assayed at 25°C but enzyme assayed at 35°C although not previously exposed to that temperature was so labile as to lose its activity *in vitro* instantaneously and have no detectable activity. The lack of activity was also found in cells of type I that were arrested for 24 h at 33°C and assayed for activity at 35°C. The rapid *in vitro* loss of enzyme activity in extracts of the parental phenotype after repeated back cross (type I) indicates that a protein directly involved in adenylate cyclase catalytic activity has been mutated. This sort of evidence from yeast cells has been taken to indicate that the structural gene for adenylate cyclase enzyme has been mutated (Matsumoto, 1982) and it is likely that this

enzyme has been directly affected in mutant 92. There is no immediate loss of activity *in vitro* by the adenylate cyclase activity in mutants 194 and 195 however after growth at 33°C the activity is only 5% of that of wild-type cells and this could indicate that the adenylate cyclase protein has been directly affected in these mutants also, but causing a much less rapid acting loss of activity. Alternatively the mutations reducing adenylate cyclase activity in mutants 194 and 195 may have altered a protein with which the enzyme associates. Certainly neither mutant 194 nor 195 retains high level of extractable adenylate cyclase activity in cells incubated at 33°C. In the case of 194 type I (parental type) the enzyme activities are less than 6% of the wild-type activity when assayed at both 25°C and 35°C. In 195 type I (parental type) adenylate cyclase activity is significant at 21°C being 27% of the wild-type but this activity is retained during 10 min of incubation at 35°C *in vitro* and is lost during *in vivo* incubation of cells at 33°C when it declined to zero.

An additional influence on the enzyme is suggested by adenylate cyclase activities measured in extracts of type II cells also obtained by backcross of mutant 92. The type II cells have presumably retained most of the genes that were present in the original isolate because they arrest in liquid at 33°C but they have not retained the ability to be rescued by exogenous cAMP. Presumably a rescue gene has been segregated to sister cells and lost from the combination of genes present in progeny of type II arrest. The rescue gene might have promoted more rapid penetration of cAMP into cells but an alternative explanation is indicated by its effect on adenylate cyclase activity. Unexpectedly the type II cells had lower adenylate cyclase activity at 25°C than type I cells although both arrest in liquid at 33°C. The activity was not detectable in type II although it is 1.8 pmol/min/mg protein in type I. The rescue gene, which is presumably absent from type II since they cannot be rescued by cAMP, is therefore related to adenylate cyclase because it resulted in an increase of extractable adenylate cyclase in type I cells cultured at 21°C and assayed for activity at 25°C. It should be mentioned that an extract of the enzyme from the type II cells at 21°C when assayed at 25°C contains no detectable adenylate cyclase but when assayed at 35°C does unexpectedly show slight activity, however this was only 7% of activity in wild-type cells. The molecular basis of this remains to be determined.

In view of the higher activity detectable in extracts from cells with phenotype I compared with phenotype II it would be interesting to test whether the capacity for rescue of adenylate cyclase activity, which is present in type I arresting cells, can also operate *in*

vitro by activation of adenylate cyclase from type II cells with extract from type I cells. This form of *in vitro* reconstitution has been possible in extracts from budding yeast since mixtures of extract from a mutant adenylate cyclase (*CDC35*) with extract from cells with mutant regulatory protein (*CDC25*) results in the recovery of fully active adenylate cyclase, presumably by association of the normal cyclase in one extract with normal *CDC25* product in the other (Engelberg et al., 1990)

Since only one extra gene confers the property of rescue and since the gene results in altered adenylate cyclase activity it is unlikely that rescue involves altered rate of cAMP uptake as well because that would require an unlikely combination of function from one gene product. However it would be interesting to test for possible alteration in the rate of uptake of radioactive cAMP due to the presence of the rescue gene.

There is no difficulty in proposing that more than one gene product can influence adenylate cyclase activity. The budding yeast adenylate cyclase is known to be influenced by close association with the *CDC25* gene product (Engelberg, Simchen and Levitzki, 1990) and by *SRV2* gene product that is necessary for full activity (Fedor-Chaiken et al., 1990) and the purified enzyme contains not only a 200 kD protein but also a 70 kD cyclase associated protein (CAP) (Field et al., 1990).

Testing for loss of activity *in vitro* in extracts transferred to 35°C immediately before assay, provides a stringent test for extreme thermolability of adenylate cyclase, but to fully test the possibility of that loss of activity developed *in vivo*, perhaps more slowly or by interaction with other adjacent proteins, extracts were taken from mutant cells that had been held at 33°C and in the case of type I and II become blocked in division. To investigate whether the prolonged exposure of whole cells to the restrictive temperature might result in changes in enzyme activity, the type II arresting progeny from back crosses of mutant 92 were shifted from asynchronous culture at 21°C to 33°C and the activity was monitored at 35°C (Fig 7.1). Although immediately on transfer an activity of 0.2 pmol/min/mg protein was detectable it declined to nondetectable levels in later extracts from the same cells when assayed in the same way. Therefore even the basal levels of activity (0.3 unit) indicated by an immediate assay at 35°C may be lost from cells held at 33°C.

To investigate whether the loss of activity *in vitro* was a secondary consequence of death of cells at the non permissive temperature samples of culture were taken at intervals and plated at 21°C to determine the number of cells remaining viable and therefore capable

of forming colonies. Cells remained viable long after extractable adenylate cyclase had become nondetectable (Fig. 7.1). Clearly the loss of activity is not a secondary consequence of cell death but rather a primary effect of a temperature conditional mutation, which then resulted in division arrest.

It was not only in mutant 92 but also in 194 and 195 that the correlation between blocked division and low adenylate cyclase activity was found. In mutant 194 adenylate cyclase activity of cells type I assayed at 35°C had 4 fold lower activity than cells of type III which were not arrested in liquid. Similarly, mutant 195 cells of type II had no activity when assayed at 35°C.

In mutant 195, cells with type I arrest (as in the original isolate) grew at 21°C and when assayed for adenylate cyclase activity at 25°C and 35°C, had almost equivalent activity. This indicated that the adenylate cyclase enzyme in this cell type was more stable to brief incubation *in vitro* at 35°C than enzyme from type I cells of 194 and 92. However, after prolonged exposured of cells to 33°C (for 24 h) no adenylate cyclase activity was detected, thus correlating with division arrest.

Taking these results together it was concluded that the defective control of cAMP accumulation in the cAMP-requiring mutants was caused by thermolability of adenylate cyclase activity and this defect consistently correlated with a block in cell division.

Table 7.3 Phosphodiesterase activities of wild-type and mutant cells at 25°C and 35°C

Cells were grown in TAPYPP medium at 21°C until they reached exponential phase then a portion of the culture was shifted to 33°C. After 24 h cells were harvested and homogenized by vortex mixing with glass beads in the solution containing 25 mM tris pH 7.5 and 1 mM PMSF and centrifuged using very low speed (1000 rpm, 5 min). The supernatant was then assayed for phosphodiesterase activity. The assay was carried out in the presence of 40 mM tris-HCl pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 60 µg BSA, 1 mM $CaCl_2$, 1 µg sheep brain calmodulin, 20 µM cAMP and 30,000 cpm [^{3}H]cAMP (specific activity 41.7 Ci/mmol). The reaction was initiated by adding 20-50 µg protein of crude cell extract to the temperature equilibrated assay mixture and incubated at 25°C or 35°C for 15 min then stopped by dipping the reaction tube into a boiling water bath for 2 min. 20 µl of snake venom *(Ophiophagus hannah)* (2 mg/ml) was added and further incubated at 30°C for 10 min. Adenine was separated from other charged products by mixing the slurry of 1 part anion exchange resin (AG-1 x 2, 200-400 mesh) and 3 part 100% methanol. The ^{3}H level in the supernatant was then determined.

Histograms were derived from data in the Table 7.3.

- represents activity of cells grown at 21°C and assayed for the activity at 25°C.
- represents activity of cells grown at 21°C and assayed for the activity at 35°C.
- represents activity of cells grown at 33°C and assayed for the activity at 35°C.

Activity units are pmol/min/mg protein.

Table 7.3

Code	Cell types	Cells 21°C assay 25°C (pmol/min/mg)	Cells 21°C assay 35°C (pmol/min/mg)	Cells 33°C assay 35°C (pmol/min/mg)
A	wild-type	532	747	679
B	92 (I)	513	743	523
C	92 (II)	601	985	534
D	92 (III)	468	807	415
E	194 (I)	522	849	408
F	194 (III)	644	937	467
G	195 (I)	797	902	619
H	195 (II)	522	849	388

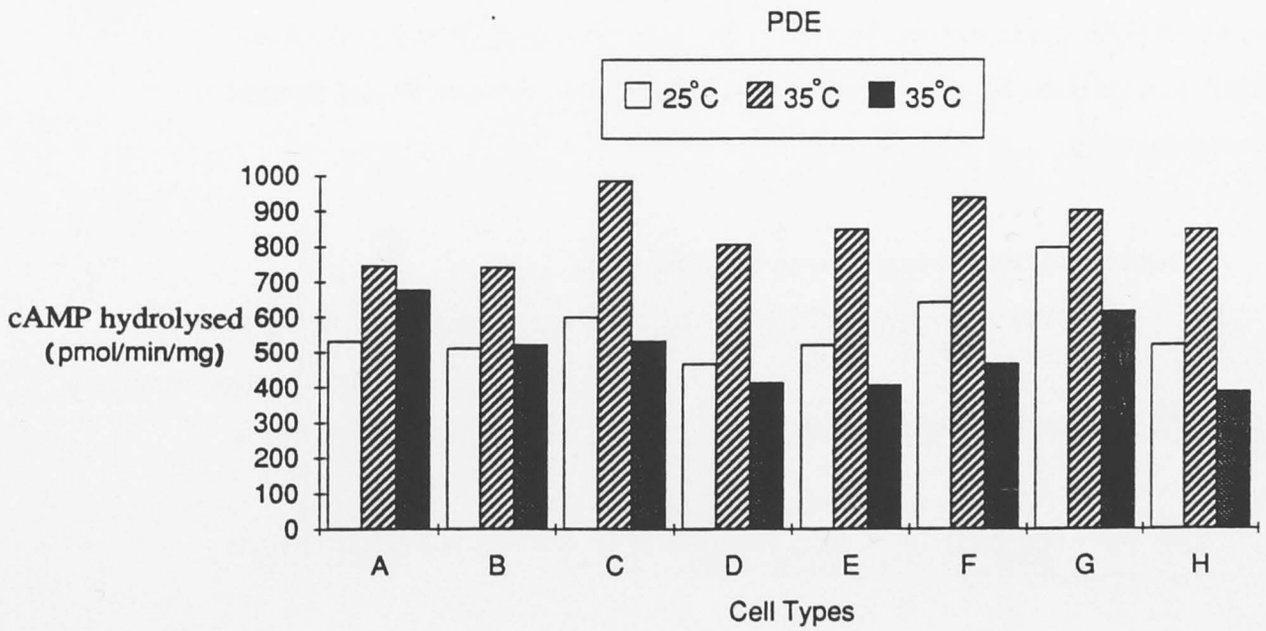


Table 7.4 Adenylate cyclase activities of wild-type and mutant cells measured at 25°C and 35°C

Cells were grown in TAPYPP medium at 21°C until they reached exponential phase then a portion of the culture was shifted to 33°C. After 24 h cells were harvested and homogenized by vortex mixing with glass beads in 25 mM tris pH 7.5 containing 1 mM PMSF and centrifuged using very low speed (1000 rpm, 5 min). Supernatants were used for adenylate cyclase assay. The extract of cells cultured at 21°C was assayed for activity at 25°C and 35°C, and extract of cells cultured at 33°C was assayed for activity at 35°C.

Adenylate cyclase was assayed in the presence of 50 mM tris pH 7.5, 300 µM cAMP, 0.1 mg/ml BSA, 0.1 mM EGTA, 0.1 mM EDTA, 20 mM creatine phosphate, 400 µg/ml creatine kinase, 1 M DTT, 10 mM $MgCl_2$, 5 mM caffeine, 0.25 mM ATP and 1 x 10^6cpm [α^{32}P]ATP specific activity 3000 Ci/mmol. [^{3}H]cAMP 5000 cpm (specific activity 41.7 Ci/mmol) was added as recovery marker. The reaction was initiated by adding 50 µg supernatant protein to assay tubes equilibrated to temperature and reaction was carried out at 25°C and 35°C for 10 min. Reaction was stopped by adding 2% SDS, 45 mM ATP, 1.3 mM cAMP. cAMP was isolated by sequential chromatography on Dowex-50 and neutral alumina columns.

Histogram was derived from data in the Table 7.4.

- ☐ represents activity of cells grown at 21°C and assayed for activity at 25°C
- ▨ represents activity of cells grown at 21°C and assayed for activity at 35°C
- ▩ represents activity of cells grown at 33°C and assayed for activity at 35°C

Activity units are pmol/min/mg protein.

Table 7.4

Code	Cell types	Cells 21°C assay 25°C (pmol/min/mg)	Cells 21°C assay 35°C (pmol/min/mg)	Cells 33°C assay 35°C (pmol/min/mg)
A	wild-type	4.7	4.2	7.6
B	92 (I)	1.8	0	0
C	92 (II)	0	0.3	0
D	92 (III)	1.73	2.7	4.1
E	194 (I)	0.2	0.4	0.4
F	194 (I)	0	0.5	0
G	194 (III)	0.6	1.6	1.0
H	195 (I)	1.3	1.2	0
I	195 (II)	0.3	0	0

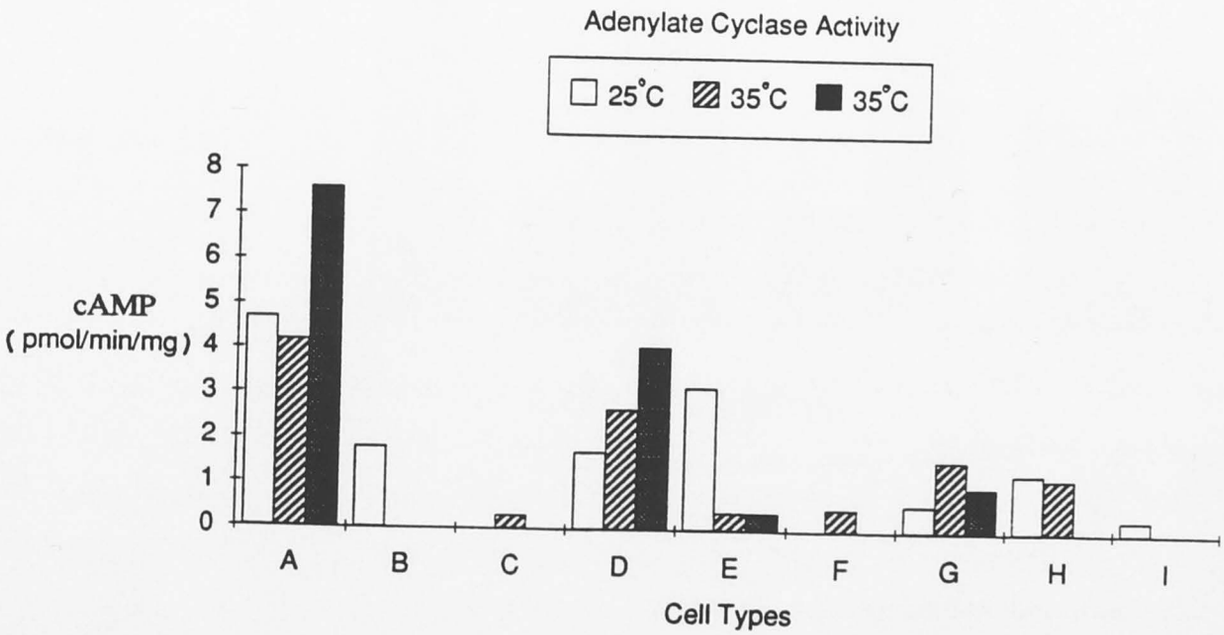
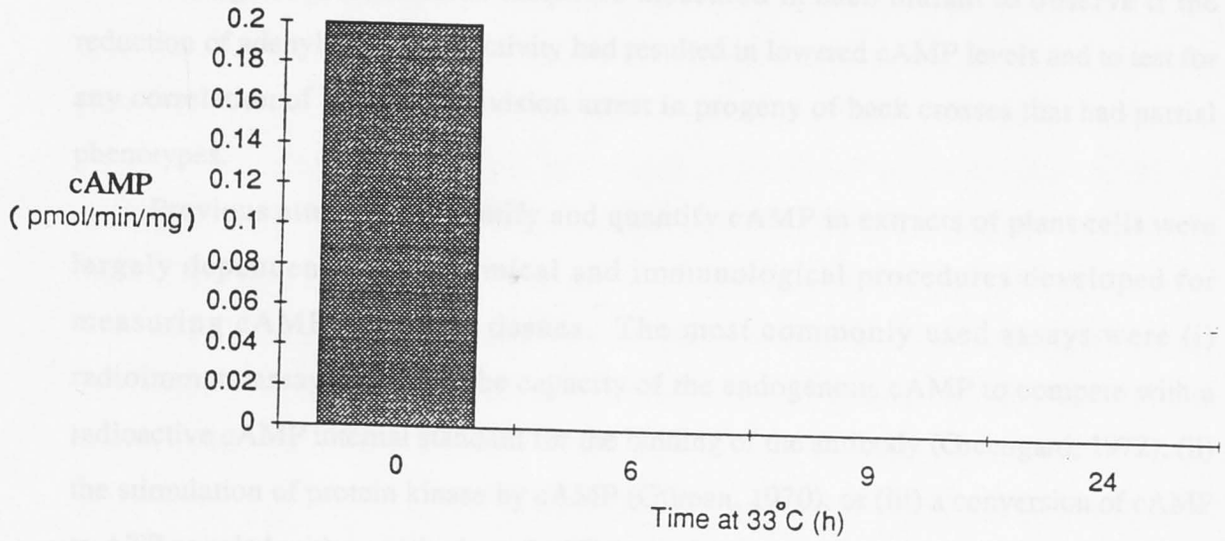


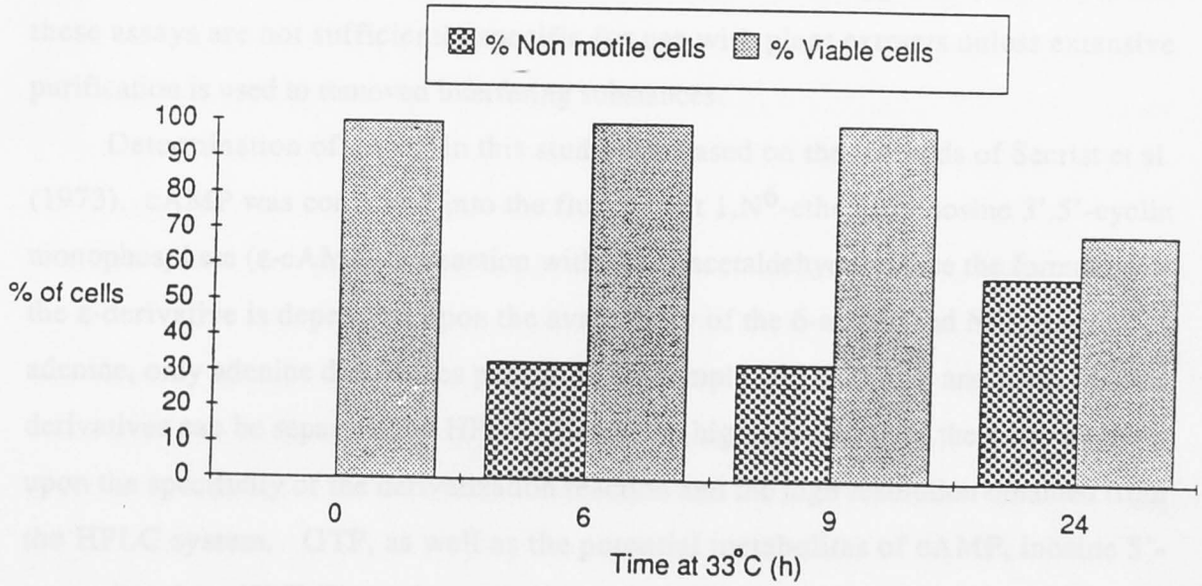
Fig. 7.1 Adenylate cyclase activity and viability of cells on transfer to 33°C.

Type II cells of mutant 92, which were derived from repeated back crossing, were cultured at 21°C in TAPYPP medium until culture reached exponential phase then cells were shifted to 33°C. Adenylate cyclase activity was assayed at 35°C immediately at the time of shifting (0 h) and then activities were monitored after cells were cultured at 33°C for 6, 9 and 24 h. Viability of the cells were observed by both counting % of non motile cells from fresh preparation and also plating onto TAPYPP plate and incubated under continuous light at 21°C for 7 days.

Time course of loss of adenylate cyclase activity in asynchronous cells of 92 transferred to 33°C



Viability of asynchronous cells of 92 on transferred to 33 C



SECTION VIII cAMP CONTENT IN ts-cAMP-REQUIRING CDC MUTANTS

The capacity of the mutants to be rescued by cAMP (section III) and their reduced levels of adenylate cyclase activity (section VII) indicated that their cAMP level might be low. Endogenous cAMP was therefore measured in each mutant to observe if the reduction of adenylate cyclase activity had resulted in lowered cAMP levels and to test for any correlation of level with division arrest in progeny of back crosses that had partial phenotypes.

Previous attempts to identify and quantify cAMP in extracts of plant cells were largely dependent on biochemical and immunological procedures developed for measuring cAMP in animal tissues. The most commonly used assays were (i) radioimmunoassay, based on the capacity of the endogenous cAMP to compete with a radioactive cAMP internal standard for the binding of the antibody (Greengard, 1972); (ii) the stimulation of protein kinase by cAMP (Gilman, 1970); or (iii) a conversion of cAMP to ATP coupled with quantitation of ATP by bioluminescense ; fluorescense (Johnson et al., 1970). In animal tissue extracts the immunoassay was often used for measuring cAMP because of its high sensitivity. However it has been suggested (Lin, 1974) that these assays are not sufficiently specific for use with plant extracts unless extensive purification is used to removed interfering substances.

Determination of cAMP in this study was based on the methods of Secrist et al. (1973). cAMP was converted into the fluorescent $1,N^6$-ethenoadenosine 3',5'-cyclic monophosphate (ε-cAMP) by reaction with chloroacetaldehyde. Since the formation of the ε-derivative is dependent upon the availability of the 6-amino and N-1 moieties of adenine, only adenine derivatives present in the sample will fluoresce and these adenine derivatives can be separated by HPLC. Hence the high specificity of the assay depends upon the specificity of the derivatization reaction and the high resolution obtained from the HPLC system. GTP, as well as the potential metabolites of cAMP, inosine 5'-monophosphate (5'-IMP) and inosine, did not form fluorescent products because they do not contain a 6-amino group on the purine ring to form the etheno derivative with chloroacetaldehyde. ATP does not interfere with the assay because it is almost completely precipitated with $ZnSO_4$ and $Ba(OH)_2$ and its fluorescent product elutes before ε-cAMP (retention time at 1.8 min). The isomeric nucleotide 2',3'-cAMP, which

exhibits chromatographic properties in other systems that are similar to that of 3',5'-cAMP, was also eluted before ε-cAMP in the HPLC system used (retention time at 3 min)

A chromatogram illustrating the typical nucleotide profile of the cell extract is shown in Fig. 8.1. Retention time of ε-cAMP is 5.10 min.

The specificity of the method was tested by comparing the elution profile of the presumed ε-cAMP region from cell extract with the elution of radioactive authentic ε-[^{3}H]cAMP that was added to the sample as a recovery tracer. The amount of added cAMP was negligible in relation to the amount of cAMP in the sample but readily detectable by its radioactivity in fractions recovered from the HPLC column. As shown in Fig. 8.2, the ε-cAMP from the cell extract co-eluted precisely with ε-[^{3}H]cAMP.

In the present study the cAMP in wild-type vegetative cells of *Chlamydomonas* was estimated at 6.9 pmol/g wet weight for cells cultured at 21°C, and 5.75 pmol/g wet weight for cell cultured at 33°C (Table 8.1). This amount is similar to that assayed by stimulation of the activity of protein kinase (Amrhein and Filner, 1973) which was estimated at 25 pmol/g dry weight (or about 5-7.5 pmol/g wet weight if it is assumed that algal cells contain 70-80% water). However these values are lower than that estimated by Bressan et al. (1980), which was 16 pmol/g wet weight. In this latter report, cAMP was assayed by supplementation of [^{32}P]ATP to the cell culture followed by detection of assumed [^{32}P]cAMP after TLC in a fraction that co-migrated with authentic [^{3}H]cAMP. Since 2',3'-cAMP exhibited TLC and column chromatographic properties similar to 3',5'-cAMP, it is possible that this could have resulted in an over estimation of cAMP. Levels of cAMP have also been estimated in gametic cells of *C. reinhardtii* at the high level of 10 pmol/10^8 cells using radioimmunoassay (Pasquale and Goodenough, 1987). This is equivalent to 200 pmol/g wet weight, assuming that 2 x 10^9 gametic cells contribute 1 g wet weight (Harris,1988). This high basal cAMP level may correlate with an involvement of cAMP in mating, since it was found that the cAMP level increased during mating and the addition of exogenous cAMP promoted mating in gamete cells (Pasquale and Goodenough, 1987).

It was also observed here that the addition of the phosphodiesterase inhibitors, caffeine and theophylline, resulted in an increased level of endogenous cAMP. The addition of 3 mM theophylline showed a greater effect in this regard than 3 mM caffeine (Table 8.1). This elevation of cAMP is in agreement with a previous report that inhibition

of phosphodiesterase elevates intracellular levels of cAMP in *C. reinhardtii* (Amrhein and Filner, 1973).

The level of cAMP determined in the three mutants is shown in Table 8.1. Cells derived by back cross from mutant 92 that retained all the genes necessary to give the arrest phenotype I (normal division at 21°C but block at 33°C unless supplemented with cAMP or db-cAMP) showed a cAMP content at 21°C of approximately 3.5 pmol/g wet weight, whereas cAMP in cells arrested at 33°C was not detectable. This result is in agreement with the finding of significant adenylate cyclase activity at 21°C, which was 38% (1.8 pmol/min/mg) of adenylate cyclase of wild-type cells (Table 8.1), and no detectable adenylate cyclase activity at 33°C. Thus cells giving an arrest phenotype I, which retained the mutant genes that were responsible for division arrest in mutant 92 in a wild type genetic background, could not produce detectable quantities of cAMP at 33°C and this correlates with a block of cell division.

In cells similarly derived by back cross from mutant 92 but retaining only the mutant genes giving arrest phenotype II, which is a block in division at 33°C with or without supplementation with cAMP, the level of cAMP at 21°C was at 1.7 pmol/g wet weight at 21°C and was not detectable at 33°C. These low levels correlate with greatly reduced adenylate cyclase activities (Table 8.1) and again absence of cAMP in the cells correlates with failure to complete the cell cycle; that is, type II arresting cells from mutant 92 at 33°C did not accumulate cellular cAMP and their division was blocked. Supplementation with cAMP did not rescue these cells to normal division since the gene combination in type II arrest did not allow rescue by exogenous cAMP. Measurements of cAMP in cells type II compared with type I arresting cells do not throw light on the reason why type II cells cannot be rescued at 33°C, because both arrest phenotypes correlate with undetectable levels of cAMP at 33°C. However there is a possible correlation of reduced cAMP level with the slow growth at 21°C of type II arresting cells, which grow at about one quarter of the rate of type I cells as measured by increase in cell number (section VI). The type II cells at 21°C have only half the cAMP content of type I cells at 21°C and only a quarter that of wild-type cells and therefore maybe limited for growth at 21°C, as well as blocked in division at 33°C when cAMP falls below a critical level.

In cells derived by back cross from mutant 92 and retaining only the genes giving arrest phenotype III, which divide normally at both temperatures in liquid medium but arrest at 33°C on agar plates, the cAMP content of cells at 21°C was 3.13 pmol/g wet

weight and cAMP content of cells cultured at 33°C in liquid medium was 5.04 pmol/g wet weight. These values are only a little lower than wild-type, which were 6.9 and 5.75 pmol/g wet weight respectively. The small reduction of cAMP in type III cells is associated with the persistence of adenylate cyclase activity to about half wild-type, whereas type I and II arrested cells have no detectable adenylate cyclase and have no detectable cAMP level (Table 8.1). There is also a more detailed correlation between the slightly higher activity of adenylate cyclase from cells of type III at 33°C, which is 4.1 pmol/min/mg protein, compared with 1.73 pmol/min/mg protein of cells at 21°C, and the level of cAMP which is 5.04 and 3.13 pmol/g wet weight at 21°C and 33°C respectively (Table 8.1). The smaller reduction of cAMP content in cells in liquid medium also correlates with their failure to arrest in liquid medium and maintains the correlation of low cAMP level with division block. These type III cells were not blocked in division in liquid presumably because they were able to produce enough cAMP, unlike the type I and type II partial phenotypes that derive from the same mutant.

In mutant 194, similar correlations of cellular cAMP levels and adenylate cyclase activities were observed in progeny types I and III that derived from back cross. In type I progeny of mutant 194, cells at 21°C contained cAMP at 2.1 pmol/g wet weight while cells at 33°C contained only 0.3 pmol/g wet weight and were arrested in division. The low cAMP content of cells at 21°C compared to wild-type was due to low adenylate cyclase activity in mutant 194 which was present at 4% of activity in wild-type cells (Table 8.1). Although at 33°C adenylate cyclase activity was slightly higher than at 21°C, these levels were only 4% of wild-type cultured at the same temperature and cells contained cAMP only at 0.3 pmol/g wet weight at 33°C. Therefore this low cAMP level again correlates with low adenylate cyclase activity at 33°C and a block in cell division. In type III progeny of mutant 194, cells at 33°C have a much higher adenylate cyclase activity compared to cells at 21°C (Table 8.1) and this resulted in higher cAMP level of cells at 33°C than at 21°C and absence of division block. Therefore again cell types derived from this different mutant conform to the three-way correlation of low adenylate cyclase with low cellular cAMP levels and arrest of division.

In mutant 195, cells of type I derived by repeated back cross, showed higher cAMP level at 21°C than did cells at 33°C; as was found for the type I arresting progeny of mutants 92 and 194, and adenylate cyclase activities of these cells correlated with this result (Table 8.1). In 195 type II cells, cAMP was detectable in cells grown at 21°C (5.9

pmol/g wet weight) while it was not detectable in cells grown at 33°C (Table 8.1). Again the three-way correlation between adenylate cyclase, cAMP level and arrest was maintained.

In conclusion the cellular cAMP content in each of the mutants was correlated with the activity of adenylate cyclase, and blocking of cell division in liquid medium correlated with both low adenylate cyclase activity and low endogenous cAMP level.

Table 8.1 cAMP content (pmol/g wet weight) and adenylate cyclase activity of wild-type and mutant cells cultured at 21°C and 33°C

cAMP was extracted by treating cells with 0.3 N PCA for 1 h then neutralized with 0.5 N $KClO_4$. The supernatants were concentrated under reduced pressured at 40°C and cAMP was purified by passing the cell extracts through neutral alumina and then Dowex-50 columns. Eluates were evaporated to dryness under reduced pressure at 40°C, then rehydrated in 400 µl of distilled water. 300 µl of the cell extract was mixed with 30 µl of chloroacetaldehyde. Reactions were carried out at 90°C for 45 min. Etheno-adenosine formed from this reaction was separated using a reverse phase HPLC (Waters) with a constant flow rate of 3 ml/min of solvent (0.05 M dibasic sodium phosphate and 0.05 M citric acid pH 4.8, 15% methanol and 26 µM cetyl pyridium Bromide). The etheno-derivatives were detected using a fluorescence detector (Shimadzu, Model RF-535; emission 410 nm, excitation 298 nm). Adenylate cyclase determinations are described in section VII. Adenylate cyclase at 21°C refers to cells incubated at 21°C and assayed for activity at 25°C. Adenylate cyclase at 33°C refers to cells incubated at 33°C and assayed for activity at 35°C.

cAMP content is expressed as pmol/g wet weight.

Adenylate cyclase activity (derived from Table 7.4) is expressed as pmol/min/mg protein.

ND = not detectable

- = not determined

caff = caffeine

theo = theophylline

Table 8.1

Cell type	cAMP content at 21°C (pmol/g wet weight)	Adenylate cyclase at 21°C (pmol/min/mg)	cAMP content at 33°C (pmol/g wet weight)	Adenylate cyclase at 33°C (pmol/min/mg)
wt	6.9	4.7	5.75	7.6
wt + 3mM caff	9.6	-	-	-
wt + 3mM theo	20.8	-	-	-
92(I)	3.5	1.8	ND	0
92(II)	1.7	0	ND	0
92(III)	3.13	1.73	5.04	4.1
194(I)	2.1	0.2	0.3	0.4
194(II)	-	-	-	-
194(III)	3.83	0.6	10.6	1.0
195(I)	8.0	1.3	2.96	0
195(II)	5.9	0.3	ND	0
195(II)	-	-	-	-

Fig.8.1 Chromatogram illustrating typical nucleotide profile of the cell extract

a. Typical profile of cell extract without adding etheno-cAMP standard. Etheno-cAMP was eluted at retention time of 5.10 min.

b. Typical profile of cell extract with addition of etheno-cAMP standard. Etheno-cAMP was eluted at retention time of 5.10 min.

8.1

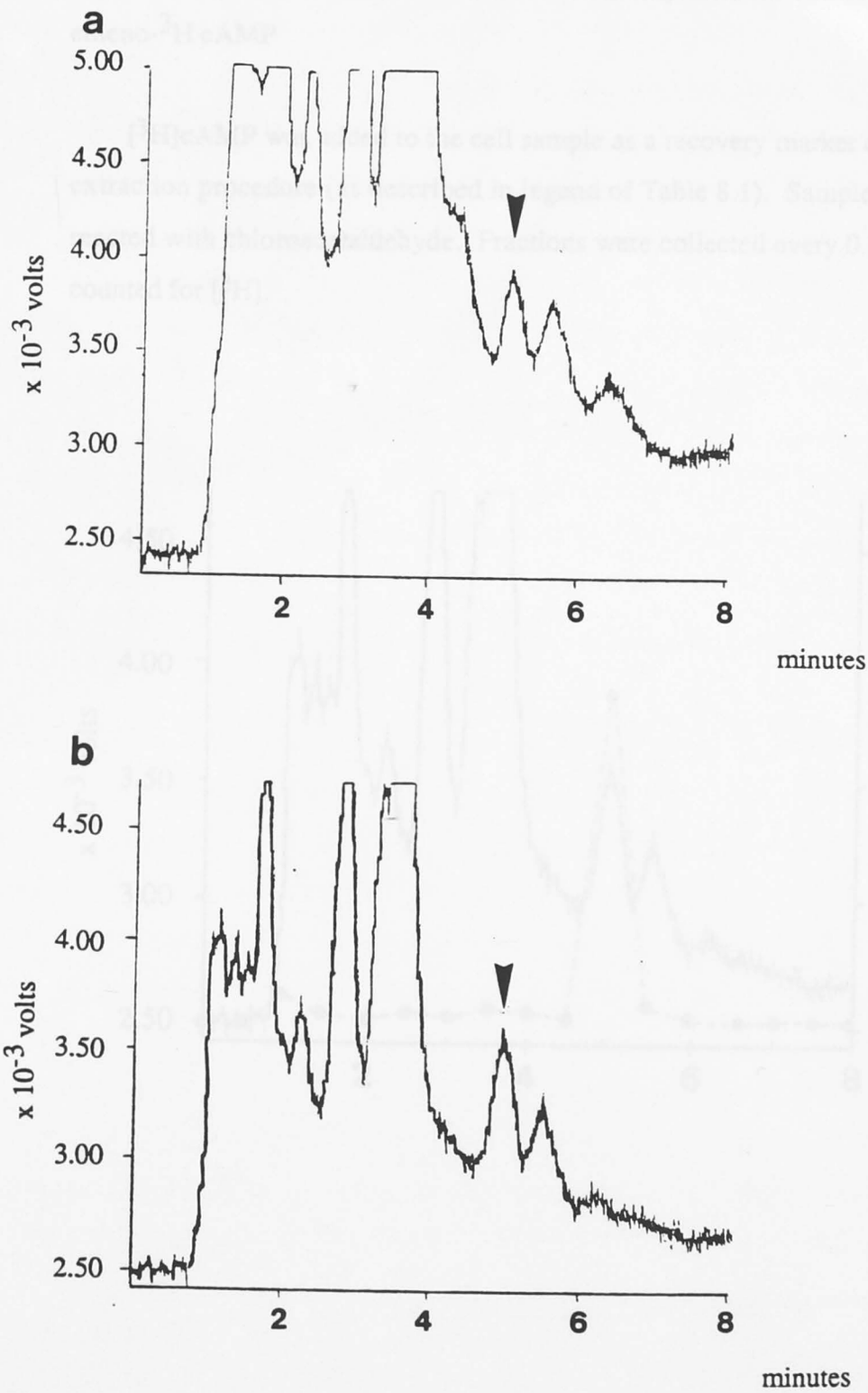
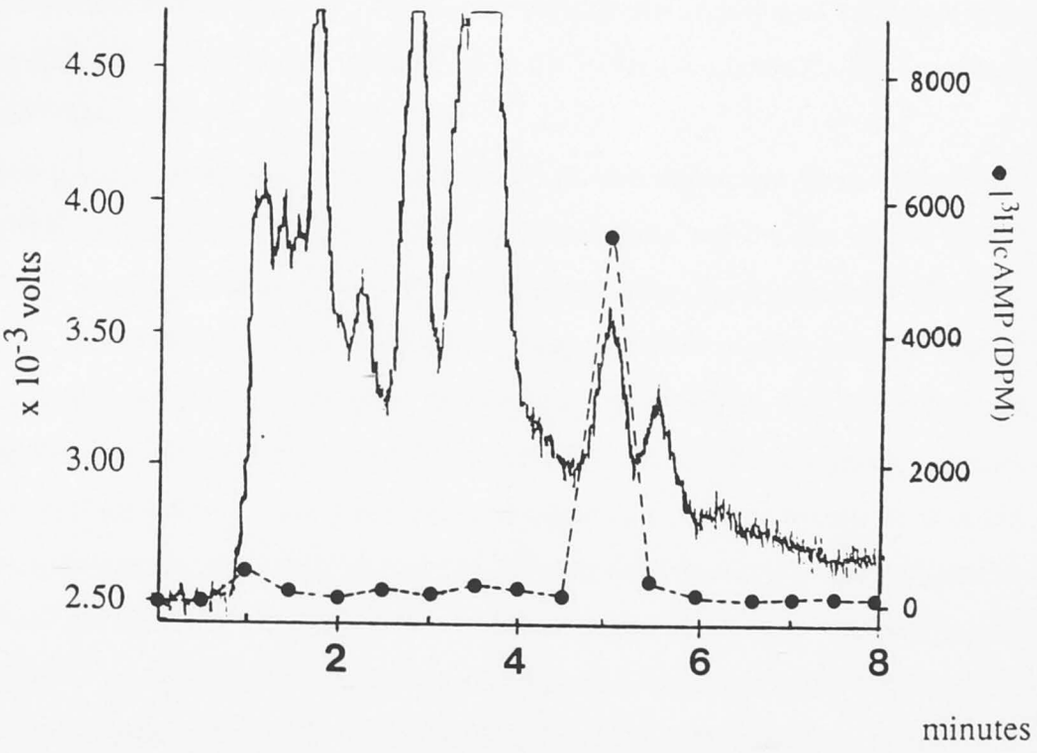

a
5.00
4.50
4.00
3.50
3.00
2.50
x 10-3 volts
2
4
6
8
minutes
b
4.50
4.00
3.50
3.00
2.50
x 10-3 volts
2
4
6
8
minutes

Fig.8.2 Co-elution of etheno cAMP from *Chlamydomonas* and of authentic etheno-^{3}H cAMP

[^{3}H]cAMP was added to the cell sample as a recovery marker during the extraction procedure (as described in legend of Table 8.1). Sample was then reacted with chloroacetaldehyde. Fractions were collected every 0.5 min and counted for [^{3}H].



GENERAL DISCUSSION

SECTION IX GENERAL DISCUSSION

It is now widely recognized that cAMP plays an important role in the regulation of many biochemical processes. There is no universal consensus concerning whether cAMP participates directly in the cell cycle nor of its possible role in the process. Several reports have noted changes in the level of cAMP in synchronous cultures but there is a difficulty concerning this form of evidence. It is possible that any changes of cAMP level are involved in adjustments of metabolism that accommodate changes in the environment during synchronization. For example, measurements of cAMP have been made in diatoms that have been synchronized by silicon starvation (Borowitzka and Volcani, 1977), *Tetrahymena* synchronized by heat shock (Dickinson, Graves and Swabda, 1976), *Chlamydomonas* synchronized by periodic photosynthetic growth and dark starvation (Sharaf and Rooney, 1985) and mammalian cells by serum starvation and refeeding (Pastan and Perlman, 1972).

Clearly these synchronizing protocols could cause perturbation of metabolism and growth that might incidentally alter cAMP levels or indeed require changes in cAMP levels to initiate adaptive response to accommodate the environmental changes. Furthermore, given the fact that a succession of the cell cycle phases is recognized in synchronous cultures, there is no time at which change in cAMP levels can be observed without its being possible to note a temporal correlation with a cell cycle phase, although this may be entirely coincidental. Nonetheless changes in cAMP continue to be detected in synchronous cultures that have been prepared under conditions using the minimum perturbation. An example is the use of an elutriator rotor, in which cells sediment against a counterflow of growth medium but are not pelleted and therefore continue uninterrupted growth. By this procedure small cells can be selected that are suitable as an early G1 inoculum for a synchronous cultures (Smith, Dickinson and Wheals, 1990).

Although any synchronizing protocol could be responsible for perturbing cAMP level it is interesting to note that, in spite of the diversity of cell types studied and the variety of experimental methods, some reviewers have recognized consistent patterns of cAMP change in relation to cell cycle phase, which could indicate that there are conserved functions of maxima or minima of cAMP level in initiating or terminating some cell cycle phases in all eukaryotes. Boynton and Whitfield (1983) noted that variation in cAMP level had been observed during the cell division cycle of microorganisms such as diatoms,

Tetrahymena pyriformis and in cells from multicellular organisms such as annelid blastema cells, regenerating amphibian limb cells and mammalian cells. All these studies indicated two peaks of cAMP in the cell cycle. The first peak of cAMP, occurring during G1, is proposed as part of an elaborate programme of events leading to DNA synthesis, whereas the second peak is observed during G2, which may correlate with the onset of mitosis (Boynton and Whitfield, 1983; Whitfield, 1987). Data from other sources can be interpreted to fit this pattern since in light/dark synchronized *Euglena gracilis*, the first cAMP increase occurred when cells were in G1 phase (Edmunds, 1964) and a second peak when cells were approaching division (Carre, et al.,1989).

One possibility is that high cAMP levels support progress through G1 phase and a decline in cAMP is part of the mechanism leading to commitment to division and progress to S phase (Boynton and Whitfield, 1983). The second peak of cAMP level might promote progress through G2 and a decline in cAMP would initiate mitosis (Carre et al., 1989). It would be an oversimplification to suppose that cAMP levels are always high in cells actively progressing through G1 phase or G2 phase. It is also not clear whether a decline in cAMP triggers START or mitosis or whether passage through this control point requires high levels and is soon followed by a decline.

Some quiescent cells have been found to have high cAMP levels. Pastan et al. (1975) have described the correlation between high levels of cAMP and the quiescent G0 state of mammalian cells, and a decline in cAMP levels in late G1, which could suggest that low cAMP levels indicate optimum growth conditions that are favourable for progress to division. This correlates with observations of decreased cAMP when quiescent mammalian cells were stimulated with serum (Bannai and Sheppard, 1974). There is a parallel with the prokaryote system in which high cAMP signals carbon starvation (Pastan and Perlman, 1972) A correlation with the behavior of mammalian cells is evident in the taxonomically-remote cell type *Tetrahymena pyriformis* in which the transition from G0 to G1 is accompanied by a decline in cAMP level (Dickinson et al., 1976). The decline in cAMP in late G1 that may contribute to initiation of DNA synthesis is positively indicated in mammalian cells by the ability of added cAMP to prevent progress from G1 to S phase (Bombik and Burger, 1973).

However, Steinberg et al. (1978) have isolated mutant S49 cells that have no detectable cAMP-dependent protein kinase activity which could indicate that these cells can

proliferate without any contribution of cAMP to cell cycle progress, if it is considered that cAMP effects can only be mediated by protein kinase A. Baserga (1985) has considered this to be particularly significant evidence against an important role of cAMP in the cell cycle. Interpretation of mutant S49 must be undertaken with caution because determination of cAMP-dependent protein kinase activity of S49 cells was observed using only histone 2B as a substrate (Steinberg et al., 1978). Failure to find cAMP-dependent protein kinase activity is not a necessarily conclusive evidence of the absolute absence of such activity since there are probably several hundred protein kinases (Hunter, 1987). Many are specific for particular substrate proteins and it is impossible to test all potential substrates. At least three different cAMP-dependent protein kinases are now known in budding yeast; TPK1, TPK2 and TPK3 (Toda et al., 1987b). Therefore it is possible that S49 had cAMP dependent protein kinase activity although not protein kinase A. It is also possible that the effect of cAMP on cell cycle progress may be exerted by influencing another class of protein other than protein kinase.

An approach to the study of cAMP in division that is complementary to the investigation of synchronous cultures is the injection of agents that affect cAMP level, or components in the cAMP response chain, into suitably large cells such as oocytes. Injection of the catalytic subunit of cAMP dependent protein kinase was equivalent to elevation of cAMP and inhibited nuclear division, while injection of the regulatory subunit was equivalent to a decrease in cAMP and induced nuclear division (Maller and Krebs, 1977).

An approach of broader potential application is the isolation of mutants in which cell division is dependent upon added cAMP. Together with this approach the use of synchronous cultures can be valuable if available mutants arrest cell cycle progress before the replicative phases of S, G2 and M have been started, as may currently be the case with budding yeast. Present yeast mutants provide no information concerning the possible contribution of cAMP at S or M phases. However, mutant analysis is in principle able to resolve uncertainty concerning whether changes in cAMP are merely coincidental under certain culture conditions, or at most incidental consequences of cell cycle progress, or are perhaps essential initiators of some division events, leading in their absence to specific block(s).

Genetic experiments with the budding yeast, *S. cerevisiae*, have indicated that cAMP is a positive effector in the G1 phase of the cell cycle (reviewed by Ishikawa et al.,

1985). The *cyr1* mutant, carrying a lesion in the adenylate cyclase gene, shows temperature-sensitive growth and is arrested in G1 phase at the non- permissive temperature (Matsumoto et al., 1984). The yeast *RAS1* and *RAS2* genes encode proteins homologous to mammalian ras proteins and the gene products are activators of yeast adenylate cyclase (Toda et al., 1985). A mutant strain carrying *RAS2*^{val19}, a gene with a missense mutation, contains constitutively activated adenylate cyclase and does not arrest in G1 upon nutrient starvation (Toda, et al., 1985). The *bcy1* mutants, lacking a functional regulatory subunit of cAMP- dependent protein kinase, produce unregulated cAMP-independent protein kinase and these also do not arrest in G1 upon nutrient starvation (Matsumoto et al., 1983). This observation eliminates the possibility that slow growth directly blocks progress from G1 phase and that cAMP is incidental to the start of cell division. Instead it indicates that slow growth normally depresses the cAMP level and that lack of cAMP is a signal that restrains the start of cell division. That restraint can be released even in starved cells by activation of the cyclase (Toda et al., 1985). These phenotypes therefore support the conclusion that low cAMP acts as a signal in budding yeast that promotes abstention from commitment to division and retention of the unbudded state that has better survival properties under unfavourable conditions.

It is appropriate to consider the relationship between the major cell cycle control point START, which is executed only when a cell has attained a critical size, and the controls that are disrupted in the *cyr1*, *ras* and *bcy1* mutants. Two classes of gene are involved in controlling START in yeast. START genes of class I are directly involved in the initiation of division events. Mutation in these class I genes results in a block of progress to division under conditions when the gene product is inactive, however, blocked cells do continue growth and are able to conjugate because they are not yet committed to division. Conversely, cells arrested by mutations in class II exhibit a phenotype similar to nutritionally-limited cells even in rich media. Such cells do not grow under conditions where the gene product is inactive, therefore they cannot execute START because they are too small and remain potentially able to conjugate, but do not do so unless allowed to resume growth. Therefore, START genes of class II are involved in growth rather than in division. Yeast strains carrying mutations of the adenylate cyclase gene, *cyr1* (allelic to *CDC35*, a gene in class II), require exogenous cAMP for growth. Removal of cAMP causes *cyr1* cells to arrest with the phenotype of START class II arrested cells. cAMP is therefore

necessary for growth as well as being a necessary positive signal of adequate growth for the START event, as has been argued above from the effect of *RAS2*^{val19} and *bcy1* mutations in circumventing the normal low cAMP level and low cAMP-dependent kinase in nutrient limited cells (Toda et al., 1985). The artificially elevated cAMP (Toda et al., 1985 and 1987a) or cAMP-dependent protein kinase (Matsumoto et al., 1983) prevents the normal orderly arrest of cell cycle progress in G1 phase on nutrient limitation.

These data have been interpreted to show a cell cycle role of cAMP in G1 by postulating that, the completion of START class II functions requires functional adenylate cyclase, thus progress from G1 to S phase could involve a high concentration of cAMP in late G1 as a necessary accompaniment to commitment to cell division. However, a simpler minimum hypothesis advanced by Nurse (1985) is that events do not occur until sufficient nutrient are available and a cell has attained a critical size. Therefore, if lack of cAMP delays growth to an adequate cell size the failure of START can be explain without any direct effect of cAMP on the START division process. This minimum hypothesis leaves open the possibility that the cAMP system may contribute to cell cycle progress at key stages in the division sequence S, G2 and M, and the capacity of the constitutively active adenylate cyclase mutants, *RAS2*^{val19} (Toda et al., 1985) or constitutively active cAMP-dependent protein kinase, *bcy1* (Matsumoto et al., 1983) to induce START indicates a possible direct role of cAMP may be a signal of adequate growth rate that feeds into the START division control.

The indications in yeast that high cAMP level both promotes growth and signals adequate growth conditions to the START mechanism, are not entirely supported by the evidence from synchronous cultures. Smith, Dickinson and Wheals (1990) studied cAMP levels in synchronous culture and found that, although the level was high at START in the first division cycles of previously unbudded cells, the division of a mother cell (previously budded cell) could be started without detectable cellular cAMP. The significance of this is unclear. We can discount any possibility that the reason for the dispensibility of cAMP prior to bud emergence of previously budded cells might be that they had already completed START very early in their cell cycle while significant cAMP maintained. This possibility is not plausible given that the cAMP decline began at the beginning of cell separation in the previous cell cycle and was completed before any bud emergence was detectable in the second cell cycle of the mother cells. It may be that dispensibility of cAMP in previously

budded cells is a peculiarity of the budding cell cycle and relates to the asymmetric division behavior of daughter buds that have never divided before which divide later than do mother mother cells. This difference in timing can be explained by size difference but possible biochemical differences cannot be discounted. Nurse has argued that START is an area rather than a single event and it could be that cAMP is required for one part of the START process and the state of having completed this part can be carried over by large mother cells until they execute START again early in their next cycle. It would be interesting to know whether previously budded cells, if grown slowly or held quiescent would gradually develop a requirement for cAMP before executing START, i.e., would gradually "forget" that they had previously divided.

A different and perhaps even less clear picture emerges from the data derived from cultured mammalian cells. There are numerous examples of cAMP acting as a negative effector of cell proliferation in experiments in which cAMP, db-cAMP or agents that increase intracellular levels of cAMP either by inhibiting cAMP phosphodiesterase or by stimulating adenylate cyclase, inhibit proliferation when added to the cultures of mouse adrenal tumour cells, rat bone marrow cells, human and mouse epithelial cells and mouse lymphoma cells (S49) (reviewed by Boynton and Whitfield, 1983). However, studies in lower eukaryotes such as *Tetrahymena* (Mains and Whitson, 1972) and *Euphotes crasus* (Verni and Rosati, 1987) show no inhibition of cell division by addition of cAMP, db-cAMP or adenylate cyclase inhibitor, as confirmed here with wild-type *Chalmydomonas* (section III). It is not impossible that there are special controls peculiar to metazoans in which cAMP is a negative effector. Certainly, metazoan cells have unique peptide growth factors (Deuel, 1987) and may also have evolved special functions for cAMP.

The special involvement of changes in cAMP level in progress through the replicative phases of S, G2 and M is also unclear. Studies in mammalian lymphoid cells show that cAMP is low through most of S phase followed by an increase in late S and G2 then decreasing during mitosis (Millis et al., 1974). The observation that cAMP levels are low in S phase is not consistent with studies in fibroblast and Hela cells which show high level of cAMP during S phase (reviewed by Friedman et al., 1976). Furthermore, studies in melanoma cells found that cAMP levels are highest in G2 and M phase which is in sharp contrast to studies in other cell types (Wong et al., 1974; reviewed by Friedman et al., 1976).

These claims and counter-claims of positive regulatory effects of cAMP in the cell cycle, make it impossible to propose a unifying model that might be applicable to all cells. One possible reason for the diversity of observations is the types of cell that are used in the experiments. Effects of cAMP on cancer cells could be different from those in normal cells. There are also uncertainties in experiments that measure effect of adding cAMP or db-cAMP because it cannot be assured that the nucleotides survive or penetrate the cells equally with different cell types and application times. The use of mutants that are defective in cAMP metabolism potentially provides a more accurate indication of the effects of cAMP.

This thesis describes attempts to isolate *Chlamydomonas* mutants in which the cAMP system may not have been so completely disabled as to prevent growth but may have lost the capacity for maintaining levels that are necessary for progress through the cell cycle. The studies of ts- cAMP-requiring cdc mutants isolated here implicated cAMP as a positive regulator of progress from G1 to S phase. In the absence of cAMP at the non-permissive temperature, the cytosleleton structure and DNA content of the mutants indicated that they had passed the division commitment point but were unable to progress through S phase. Defective cAMP-metabolism in these mutants is not caused by phosphodiesterase activity which was normal in all mutants, but by altered level and thermolability of adenylate cyclase activity. The cellular cAMP contents in these mutants correlated with *in vitro* measurements of adenylate cyclase activity. The abnormally low intracellular cAMP level and low adenylate cyclase activity consistently correlated with the arrest of cells in liquid medium at the non-permissive temperature.

It is not possible to conclude from these data what the pattern of change in cAMP may be through the *Chlamydomonas* cell cycle. We can conclude that a cAMP level above a certain threshold is necessary for progress from commitment to division to S phase but we cannot deduce whether this level is constant through G1 or whether it continues after commitment. It is certainly difficult to measure cAMP level during the cell cycle without perturbation of the system particularly in this photosynthetic organism. The evidence presented here does suggest that cAMP is a positive regulator of the G1 to S transition and this correlates with some of the evidence currently available from yeast.

It is not clear what the biochemical functions of cAMP may be at the G1/S transition. This control point, although in many cells more important than G2/M control, has been less thoroughly investigated. One protein that is clearly implicated at START is

the p34$^{cdc2/28}$ protein kinase whose necessity is indicated by the effect of mutation in blocking G1 to S progress (Hartwell; 1978; Nurse and Thuriaux, 1980) and by biochemical evidence that depletion of p34^{cdc2} kinase in *Xenopus* oocyte extracts prevents the initiation of DNA synthesis (Blow and Nurse, 1990). However this kinase is not dependent on cAMP when taken from either oocytes (Blow and Nurse, 1990) or from higher plant tissues (John, Sek and Hayles, 1991). Although the p34^{cdc2} kinase is not dependent upon cAMP there are presumably a number of biochemical events that follow START and are directly involved in initiation of DNA synthesis, which may be dependent on cAMP.

It should be noted that available evidence from yeast is consistent with the possibility that cAMP acts after START and prior to S phase, as is suggested by the present evidence from *Chlamydomonas*. The yeast mutant *RAS2*^{val19}, which produces high levels of cAMP, continues to progress from G1 to S phase even when cells are starved (Toda et al., 1985). This could be by cAMP promotion of START, but also could involve cAMP stimulation of an event after START. No clear indication that cAMP acts at START is given by the effect of cAMP deficient cells which do arrest prior to START because these cells are unable to grow and this failure alone could account for failure of START division. A direct effect of cAMP at START is apparently indicated by the report of Matsumoto et al.(1983) that the *bcy1* (constitutively active cAMP dependent protein kinase) mutant under nitrogen starvation "sometimes continued bud emergence for multiple cycles without further nuclear division resulting in multiple buds". If this important observation is true, it implies that cAMP does directly stimulate START and can also circumvent the normal cell size monitoring mechanism at START. If cAMP does act predominantly at the START event in budding yeast then this may be a point of difference between the yeast and *Chlamydomonas*. However, it remains possible that in both yeast and plants cAMP is necessary for START and a higher level after START is necessary for initiation of S phase. The *bcy1* mutation could promote both events, but the reduced levels of cAMP caused by the mutation isolated here could be permissive for START/commitment but not for the immediate post START events leading to S phase.

Although cAMP-requiring mutants isolated in this present work are blocked in the early stage of the cell cycle, this leaves open the possibility that cAMP has a positive role in later stages of the cell cycle. Studies in *Xenopus* oocytes indicate involvement of cAMP in the induction of nuclear division. It was found that a reduction in cAMP level or

inactivation of cAMP-dependent protein kinase in oocytes was sufficient, but not necessary to induce nuclear division (Gelerstein et al., 1988). This conclusion was indicated by microinjection of the regulatory subunit of cAMP-dependent protein kinase (equivalent to a decrease in intracellular cAMP), which accelerated progesterone-induced maturation in *Xenopus* oocytes, while the injection of catalytic subunit (equivalent to an elevation of cAMP) (Maller and Krebs, 1977) and the addition of PDE inhibitor; methylxanthines (O'Connor and Smith, 1976) and adenylate cyclase activator, forskolin (Schorderet-Slatkine and Baulieu, 1982) inhibited maturation induced by progesterone. However, not only cAMP that affects maturation process but also intracellular calcium, acetylcholine and adenosine accelerate progesterone-induced nuclear division in *Xenopus* oocytes that was accompanied by only small changes in the cellular levels of cAMP (Baulieu et al., 1978; Wasserman et al., 1980; Gelerstein et al., 1988). Although these results indicated that cAMP may not be an essential regulator in the induction of nuclear division, it certainly can do so and it is noted that this is a complex experimental system in which hormone presence is essential and in which cAMP may be acting as a modulator of the hormone induction of nuclear division.

Within mitosis there is also evidence that low cAMP promotes progress through the later stages to completion of anaphase. Events in late mitosis may be triggered by low cAMP level as reported by Smith et al (1990), who found that cAMP level was lowest prior to and just after cell separation in the cell cycle of *S. cerevisiae*. This correlates with the observation of an *S. pombe* mutant, *dis* in which the separation of sister chromatids during mitotic anaphase is coupled to the transient decrease of intracellular cAMP concentration. Since the addition of caffeine at the concentrations that do not affect growth and division of wild-type, blocks growth and division of the *dis* mutants, and also *dis* mutant carrying multicopy plasmid containing the PDE gene presumably enhancing cAMP degradation complemented the *dis* mutation, this could indicate that the *dis* mutation causes an inhibitory excess of cAMP at mitosis. Therefore this indicated that anaphase requires low cAMP and is perhaps triggered by a reduction cAMP level (Hirano, 1988).

No unifying view of the role of cAMP in the division cycle of all eukaryote cells can be derived from the available information. However, cAMP seems to be an essential molecule in the biochemical process of growth as was found in the yeast mutants, *cyr1*, and the work in this thesis supports the idea that cAMP acts as a positive regulator during

progression from G1 to S phase. The mutations in *Chlamydomonas* that are described here clearly do not act to prevent growth and therefore their blocking of division is not a secondary effect; instead it derives from a blockage of progress from G1 through S phase.

The cAMP-requiring cdc mutants isolated here could provide an important genetic resource for study of the role of cAMP in the cell cycle with clear advantages over many of the currently available yeast mutants, which predominantly affect growth and may only indirectly affect division. Furthermore identification and isolation of the cAMP-related division genes in these mutants could lead to a better understanding of their function in controlling the cell cycle and would make feasible testing for possible higher plant homologues of cAMP-related cell division cycle genes.

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